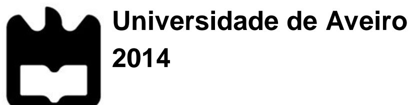

Universidade de Aveiro
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Secção Autónoma de Ciências da Saúde

Mariana da Rocha Soares Guedes

Mitocôndrias e Peroxissomas: papel na defesa celular antiviral

Mitochondria and Peroxisomes: role within cellular antiviral defense

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica da Doutora Daniela Maria Oliveira Gandra Ribeiro, PhD, Investigadora Principal do laboratório "*Organelle Dynamics in Infection and Disease*", pertencente ao Centro de Biologia Celular da Universidade de Aveiro

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Palavras-Chave

mitocôndrias, peroxissomas, MAVS, RIG-I, imunidade inata, respostas antivirais, vírus, interferão, genes estimulados por interferões

Resumo

O presente trabalho propõe-se a rever e compilar toda a bibliografia cientificamente relevante até à data, no que respeita as vias de sinalização antivirais implicadas na imunidade celular inata em células humanas.

Com ênfase na proteína adaptadora MAVS, este trabalho explora as particularidades das vias de transdução de sinal e respetivos intervenientes em dois organelos celulares específicos: mitocôndrias e peroxissomas. Estas vias, em última instância, resultam na expressão de genes estimulados por interferões (ISGs), principais responsáveis pelo combate celular eficaz contra a replicação viral, montagem de partículas virais e libertação de viriões na célula infetada.

Neste trabalho são ainda apresentadas propostas para investigações futuras, uma vez que ainda muito pouco se sabe sobre o papel dos peroxissomas nas respostas imunitárias inatas contra infeções virais.

Keywords

mitochondria, peroxisomes, MAVS, RIG-I, innate immunity, antiviral response, virus, interferon, interferon-stimulated genes

Abstract

The present paper presents a review and compilation of all the scientifically relevant bibliography to date, regarding the antiviral signalling pathways implicated in the cellular innate immune system in humans.

Emphasizing the mitochondrial antiviral signalling adaptor (MAVS), this paper explores the special features of the signal transduction pathways and their components in two specific organelles: mitochondria and peroxisomes. These pathways, ultimately, result in the expression of interferon-stimulated genes (ISGs), which are primarily responsible for fighting against viral replication, viral particle assembly and virion release within the cell.

In this paper, several proposals for further investigation are also presented, since there is still a lot to learn about the role of peroxisomes in the antiviral innate immune responses.

“Science, my boy, is made up of mistakes, but they are mistakes which it is useful to make, because they lead little by little to the truth.” — Jules Verne, Journey to the Center of the Earth

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List of Abbreviations

ACTH – Adrenocorticotropic Hormone

ADP – Adenosine Diphosphate

AIM2 – Absent In Melanoma 2

AIP4 – Atrophin-1-Interacting Protein 4

AP-1 – Activator Protein 1

Atg5-Atg12 – Autophagy protein 5 – Autophagy protein 12

ATF-2/c-Jun – Activating Transcription Factor 2/c-Jun

ATP – Adenosine Triphosphate

Bcl-10 – B-cell lymphoma/leukemia 10

c-Abl – c- Abelson murine leukemia

CARD – Caspase Activation and Recruitment Domain

Cardif – CARD adaptor inducing IFN-β

CBP - CREB-Binding Protein

CD4 – Cluster of Differentiation 4

CLR – C-type Lectin Receptor

CO_2 – Carbon Dioxide molecule

COX5B – Cytochrome C Oxidase 5B

c-Src – proto-oncogene tyrosine-protein kinase Src

CTD – C-Terminal Domain

CTLDs – C-Type Lectin-like Domains

CYLD – Cylindromatosis

DAI – DNA-dependent Activator of IFN-regulatory factor

DAK – Dihydroacetone Kinase

DAMP – Danger-Associated Molecular Patterns

DBD – DNA-Binding Domain

DCIR – Dendritic Cell Immunoreceptor

DLP1 – Dynamin-Like Protein 1

DNA – Deoxyribonucleic Acid

DUBA – Deubiquitinating enzyme A

EBV – Epstein Barr Virus

eIF-2α – eukaryotic Initiation Factor 2, subunit alpha

EMCV – Encephalomyocarditis Virus

ER – Endoplasmic Reticulum

EYA4 – Eyes Absent 4

FAD – Flavin Adenine Dinucleotide

$FADH_2$ – Flavin Adenine Dinucleotide, Reduced

FADD – Fas-Associated Death Domain

FAK – Focal Adhesion Kinase

Fis1 – Fission protein 1

FLN29 – aka TRAFD1 - TRAF-type zinc finger domain-containing protein 1

GAS – Gamma interferon Activation Sites

gC1qR – receptor for globular head domain of complement component C1q

GRX-1 – Glutaredoxin-1

H_2O – Water molecule

HCMV – Human Cytomegalovirus

HCV – Hepatitis C Virus

HIV – Human Immunodeficiency Virus

HIV-1 – Human Immunodeficiency Virus-1

HMGA1 – High Mobility Group Protein A1

HPV – Human Papilloma Virus

Hsp90 – Heat shock protein 90

HSV-1 – Herpes Simplex Virus-1

IAD – IRF-Association Domain

IFIT3 – IFN-induced protein with Tetratricopeptide repeats 3

IFN – Interferon

IFNAR – Interferon-alpha/beta Receptor

IκBα – nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha

IKK – IκB kinase

IL-1β – Interleukin-1 beta

IPS-1 – IFN-β Promoter Stimulator-1

IRF – Interferon Regulatory Factors

ISGF3 – Interferon-Stimulated Gene Factor 3

ISRE – IFN-Stimulated Regulatory Element

JAK – Janus Activated Kinase

JEV – Japanese Encephalitis Virus

JNK – c-Jun N-terminal Kinase

KSHV – Kaposi's Sarcoma-associated Herpes Virus

LGP2 – Laboratory of Genetics and Physiology-2

LRRFIP1 – Leucine-Rich Repeat Flightless-Interacting protein 1

MAL – MyD88 Adapter-Like

MALT1 – Mucosa-Associated Lymphoid tissue lymphoma Translocation protein 1

MAMs - Mitochondrial Associated Membranes

MAPK – Mitogen-Activated Protein (MAP) Kinase

MAVS – Mitochondria Antiviral Signalling

MDA5 – Melanoma Differentiation-Associated 5

MEFs – Mouse Embryonic Fibroblasts

MEKK1 – Mitogen-Activated Protein (MAP) Kinase/Extracellular signal-Regulated protein Kinase (ERK) Kinase 1

Mff – Mitochondrial fission factor

Mfn – Mitofusin

MHC - Major Histocompatibility Complex

MHV – Mouse Hepatitis Virus

MICL – Myeloid C-type Lectin-like receptor

mRNA – messenger Ribonucleic Acid

NADH – Nicotinamide Adenine Dinucleotide dehydrogenase

NAD^+ - Nicotinamide Adenine Dinucleotide +

NAP-1 – NF-kB-Activating kinase-associated Protein 1

Ndfip1 – Nedd4 family interacting protein 1

NDV – Newcastle Disease Virus

NEMO – NF-kappa-B Essential Modulator

NES – Nuclear Export Sequence

NFAT – Nuclear Factor of Activated T-cells

NF-kB – Nuclear Factor- kappaB

NLR – Nucleotide-binding oligomerization domain (NOD)-Like Receptor

NLRX1 – Nod-Like Receptor X1

NLS – Nuclear Localization Sequence

NMR – Nuclear Magnetic Resonance

NS – Non-Structural protein

O_2 – Oxigen molecule

Opa1 – Optic atrophy 1

ORF47 – Open Reading Frame 47

OTUB – OTU deubiquitinase, ubiquitin aldehyde Binding protein

PAF1 – Polymerase Associated Factor 1

PAMP – Pathogen-Associated Molecular Pattern

PCAF – p300/CBP-Associated Factor

PCBP1 – Poly(rC)-Binding Protein 1

Pex – Peroxin

PKR – Protein Kinase K

PLK1 – Polo-Like Kinase 1

pppRNA – triphosphate Ribonucleic Acid

PRDs – Positive Regulatory Domains

PRR – Pattern-Recognition Receptor

PTS – Peroxisomal Targeting Sequence

PSMA7(a4) – Proteasome subunit alpha type-7, subunit a4

RD – Repressor Domain

RHD – Rel-Homology Domain

RIG-I – Retinoic acid-Inducible Gene-I

RING – Really Interesting New Gene

RIP-1 – Receptor Interacting Protein 1

RLR – RIG-I-Like Receptor

RNA – Ribonucleic Acid

RNF – RING finger protein

RNF135 – RING finger protein leading to RIG-I activation or Riplet

ROS – Reactive Oxigen Specie

rRNA – ribosomal Ribonucleic Acid

RSV – Respiratory Syncytial Virus

SARS-CoV – Severe Acute Respiratory Syndrome coronavirus

SeV – Sendai virus

SHP –SH2-domain-containing protein tyrosine Phosphatase

SIKE – Suppressor of IKKepsilon

SINTBAD – Similar to NAP1 TBK1 adaptor

Smurf1 – SMAD specific E3 ubiquitin protein ligase 1

STAT – Signal Transducer and Activator of Transcription

STING – Stimulator of Interferon Genes

ssRNA – single-stranded Ribonucleic Acid

SWI/SNF – SWItch/Sucrose Non Fermentable

SYK – Spleen Tyrosine Kinase

TAD – Transcription Activation Domain

TAK1 – aka MAP3K7 – Mitogen-activated protein kinase kinase kinase 7

TANK – TRAF-family member associated NF-kB activator

TBK1 – TANK-binding kinase 1

TBP – TATA-binding protein

TFIID – Transcription Factor II D

TFIIE – Transcription Factor II E

TFIIF – Transcription Factor II F

TFIIH – Transcription Factor II H

TIM – TRAF-interacting motif

TIR – Toll-Interleukin-1 Receptor

TIRAP – Toll-interleukin 1 receptor (TIR) domain-containing Adaptor Protein

TLR – Toll-Like Receptor

TNFR - Tumour Necrosis Factor Receptor

Tom70 – Translocase of outer membrane 70

TRADD – Tumour Necrosis factor receptor type 1-associated Death Domain

TRAF – TNF Receptor Associated Factor

TRAM – Toll-Receptor-Associated Molecule

TRAP – Target of RNAIII-Activating Protein

TRIF – TIR-domain-containing adapter-inducing interferon-beta

TRIM25 – Tripartite Motif-containing protein 25

tRNA – transfer Ribunucleic Acid

TSPAN6 – Tetraspanin protein 6

TYK2 – Tyrosine Kinase 2

UbcH8 – a.k.a. UBE2L6 - Ubiquitin/ISG15-conjugating enzyme E2 L6

UBNX1 – Ubiquitin regulatory X domain-containing protein

vIRF – viral Interferon Regulatory Factor

VISA – Virus-Induced Signalling Adaptor

VSV – Vesicular Stomatitis Virus

WNV – West Nile Virus

ZAPS – Zinc finger Antiviral Protein Shorter isoform

1 – Introduction

1.1 – Mitochondria: general features and functions

Mitochondria are among the largest cellular organelles in the eukaryotic cell, with the same approximate size of an *E. Coli* bacterium (Lodish et al., 2008). They occupy a maximum of 25% of the cell cytosolic volume, depending on the cell type (e.g.: liver cells can have up to two thousand mitochondria, occupying a fifth of the cell volume (Alberts, 2008; Lodish et al., 2008).

Despite being large enough to be visualized under light microscopy, the details of its structure were only recognized with the use of electron microscopy and their unique functions were first revealed upon the development of experimental procedures in order to isolate intact mitochondria (Alberts, 2008; Lodish et al., 2008). The first observations of intracellular structures that could represent mitochondria date back to the 1840s. In 1890, Richard Altmann recognized the ubiquitous occurrence of these structures and named them "bioblasts". The term "mitochondrion" was coined by Carl Benda in 1898 and derives from the Greek "*mitos*" (thread) and "*chondros*" (granule), referring to the appearance of these structures during spermatogenesis (Ernster and Schatz, 1981).

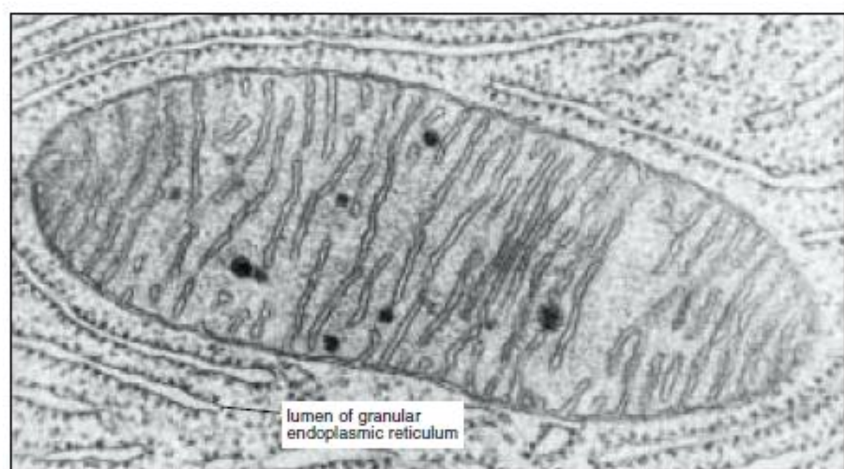

Figure 1 – Electron microscopy photograph of a mitochondrion.
(Hall, 2010)

Mitochondria are usually portrayed as stiff elongated cylinders with an approximate diameter of 0.5 to 1µm. However, live cell studies have shown that mitochondria are incredibly dynamic and plastic organelles, constantly changing their shape, even fusing

with one another and splitting up again. As they move along the cytosol, they appear frequently associated with microtubules, which might determine the orientation and distribution of mitochondria in different cell types: in cardiac muscle cells they appear to be packaged around adjacent myofibrils while, in sperm cells, mitochondria are mainly found wrapped around the flagellum (Alberts, 2008).

Each mitochondrion is surrounded by two specialized membranes that together create two separate mitochondrial compartments (*Figure 2*): an intermembrane space and a core space, named the mitochondrial matrix (Alberts, 2008). The outer membrane defines the external perimeter of mitochondria while the inner membrane has numerous convolutions and infoldings – named cristae – that greatly increase the surface area of this membrane (Lodish et al., 2008).

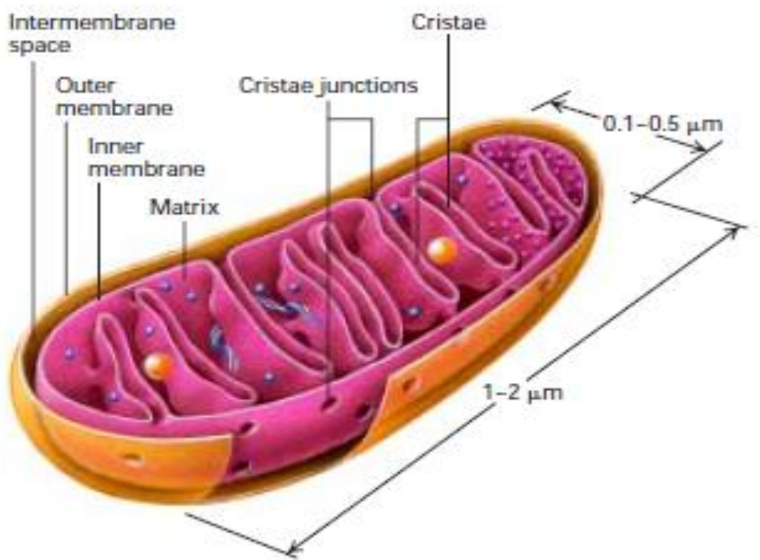

Figure 2 – Scheme of a mitochondrion with the individualized internal compartments. *Two membranes form the main structure of the mitochondrion, dividing it into three compartments: outer membrane, intermembrane space, inner membrane and matrix. The inner membrane possesses infloldings named cristae and contain ribosomes (blue spheres) facing the matrix. The matrix compartment contains, among others, the mitochondrial DNA (blue strands) and granules (yellow spheres). (Lodish et al., 2008)*

Each membrane has its own set of proteins. The outer membrane contains porins – transmembrane channels similar to bacterial porins – allowing the flow of ions, molecules and proteins no bigger that 5kDa, in and out of the intermembrane space. However, while the intermembrane space is chemically equivalent to the cytosol, the matrix is a much more selective space, due to the special features of the inner membrane (Alberts, 2008). Proteins account for ~76% of the total weight of the inner

membrane. Some of them are F_0F_1 complexes that synthesize ATP while others function in the transport of electrons, O_2 and NADH. Others allow that some otherwise impermeable molecules such as ADP and Pi enter the matrix and molecules such as ATP exit the matrix. A great proportion of the inner membrane is composed of cardiolipin that helps to keep this membrane especially impermeable to ions, reducing the membrane permeability to protons (Lodish et al., 2008).

The inner mitochondrial membrane, the cristae and the matrix are the sites where most of the reactions involving pyruvate and fatty acids oxidation into CO_2 and H_2O with ATP production take place – citric acid cycle and oxidative phosphorylation, respectively. This is a multistep process but it can be divided into three simple groups of reactions:

1. Pyruvate and fatty acid oxidation into CO_2 and combination with co-enzyme A to form acetyl Co-A, coupled with NAD^+ reduction into NADH and FAD into $FADH_2$ – **citric acid cycle**. These electron transporters are not permanently bound to proteins and are the source of electrons to the electron transporter chain; most of these reactions occur in the matrix or in the inner membrane facing the matrix.
2. Transfer of electrons from NADH and $FADH_2$ to O_2, regenerating these electron transporters back to its oxidized form NAD^+ and FAD – **oxidative phosphorylation**. These reactions take place at the inner membrane.
3. Recovery of the stored energy in the electron transport chain in order to produce ATP at the inner membrane (Lodish et al., 2008).

While most organelle proteins are coded in the nuclear genome of the cell, synthesized in ER ribosomes and then imported to the destination organelles, others, such as mitochondrial proteins, are coded by mitochondrial DNA and synthesized in ribosomes within the organelle itself (Alberts, 2008).

The human mitochondrial genome contains 2 rRNA genes, 22 tRNA genes and 13 protein-encoding sequences that are core constituents of the respiratory complexes embedded in the inner membrane. The mitochondrial DNA molecule is circular (like a typical bacterial genome) and, in mammals, it has around 16 500 base pairs, which accounts for less than 0,001% of the size of the nuclear genome. Mitochondrial genome is contained within the matrix and is usually arranged in clusters named nucleoids which are thought to be attached to the inner mitochondrial membrane. This

nucleoid organization resembles the one of bacteria and, as for bacteria, it does not possess histones (Alberts, 2008).

The number of mitochondria and their shape varies among different cell types and it can change within the cell under special physiological conditions, so that mitochondria can be presented as spherical organelles or in a single branched structure (or reticulum). This arrangement is controlled by mitochondrial fission and fusion events that are regulated by a special set of GTPases localized at mitochondrial membranes (Alberts, 2008) – the members of the dynamin family: Mitofusins (Mfn1 and Mfn2) mediate the outer membrane fusion in mammals, while Opa1 mediates fusion of the inner membrane; Drp1, which cycles between the cytosol and the outer membrane mediates mitochondrial fission (van der Bliek et al., 2013).

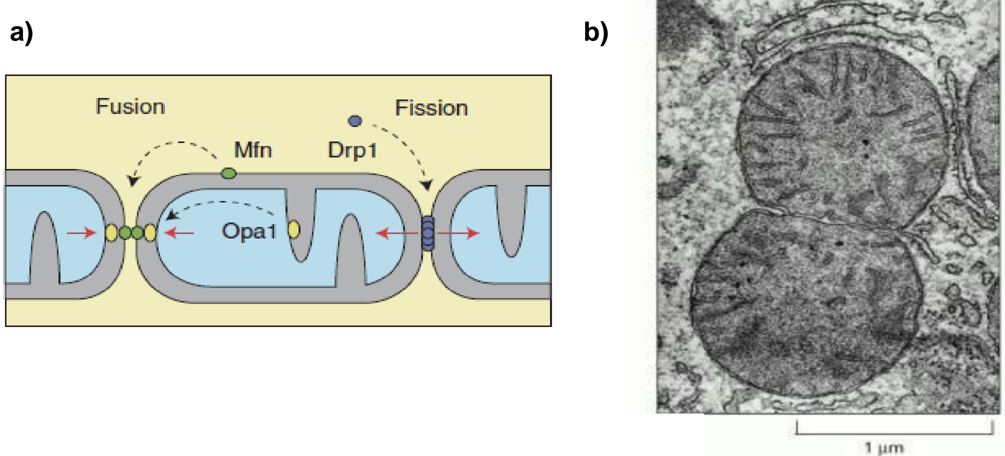

Figure 3 – Representation of the mitochondrial growth and division events.
a) Three proteins constitute the fusion/fission machinery of mitochondria: mitofusins (Mffn) mediate outer membrane fusion in mammals while Opa1 mediates inner membrane fusion; Drp1 (also known as DLP1) cycles between the cytosol and the mitochondrial outer membrane and mediates mitochondrial fission; b) Electron micrograph of a dividing mitochondrion in a liver cell. Adapted from: (Alberts, 2008; van der Bliek et al., 2013)

Despite energy conversion from glucose or fatty acids into ATP being the most significant function of mitochondria – which are commonly designated as the "powerhouse of the cell" due to this feature – these organelles are involved in a much larger number of signaling pathways involving apoptosis, immune responses, calcium homeostasis, control of the cell growth and division, etc., rendering mitochondria as all-purpose organelles (Lodish et al., 2008).

1.2 – Peroxisomes: general features and functions

Peroxisomes differ from mitochondria in some aspects, such as the fact that they are surrounded by a single membrane and do not possess their own set of DNA (Alberts, 2008). However, they are a part of the basic equipment of the eukaryotic cell and perform important metabolic functions involved in the hydrogen peroxide and fatty acid metabolism, being therefore essential to the health and development of human beings (Islinger and Schrader, 2011; Schrader and Fahimi, 2008).

Peroxisomes (originally called microbodies) were first described by a Swedish doctoral student J. Rhodin in 1954 but were only identified as organelles by the Belgian cytologist Christian de Duve in 1967. Peroxisomes were first defined as organelles where oxidation reactions take place leading to the production of hydrogen peroxide. Since hydrogen peroxide is harmful to the cell, these organelles contain an array of enzymes, particularly catalase, which decomposes hydrogen peroxide into O_2 and water molecules. A variety of substrates are broken by these oxidation reactions in the peroxisomes, including fatty acids (β-oxidation), ethanol (particularly in liver cells) and uric acid (Cooper, 2000).

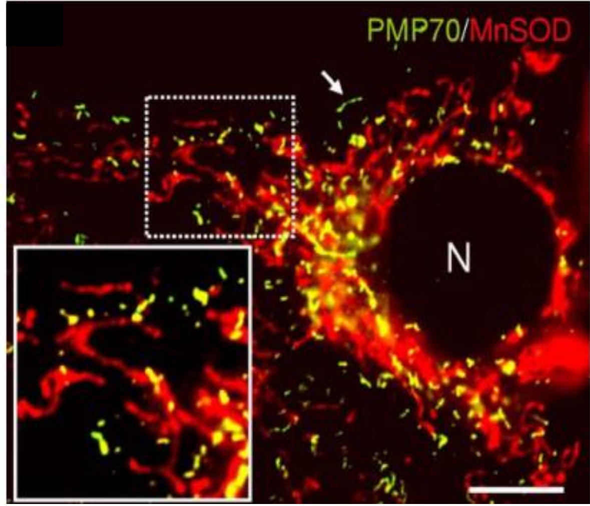

Figure 4 – Fluorescence microscopy photograph of peroxisomes (green) and mitochondria (red) in human hepatoma cells.
Peroxisomes vary in their morphology, cycling between a spherical, rod-shaped morphology and elongated tubular morphologies, while mitochondria are mainly found in the form of an interconnected tubular network. Peroxisomes can also present a "beads on a string" morphology during division events (arrow). Boxed region shows higher magnification view. N nucleus. Scale bars 10nm. Adapted from: (Schrader and Fahimi, 2008)

However, peroxisomes functions are not restricted to oxidation reactions: they are multitask organelles, with functions that vary concerning the cell type or development state of the organism. Several specialized functions have been developed such as the penicillin biosynthesis in fungi, glycolysis in trypanosomes, photorespiration and glyoxylate cycle in plants and plasmalogen biosynthesis in mammals (plasmalogens are important constituents of myelin sheets in the brain) (Islinger and Schrader, 2011; Schrader and Fahimi, 2008).

Besides catalase, peroxisomes possess other enzymes involved in the production and elimination of reactive oxygen species (ROS), such as superoxide dismutase, peroxiredoxins and glutathione peroxidase, actively participating in ROS metabolism, oxidative stress, neurodegeneration and carcinogenesis (Schrader and Fahimi, 2008).

The fatty acid oxidation is a particularly relevant function, once it provides the main source of metabolic energy to the cell (Cooper, 2000). While in yeast and plants β-oxidation of fatty acids depends exclusively on peroxisomes, in mammals this function takes place in both, peroxisomes and mitochondria. Both organelles have their own set of β-oxidation enzymes but have different specificities for certain fatty acids. Very long chain fatty acids and phytanic acid for example, can only be degraded in peroxisomes. Additionally, in mammals, peroxisomes are involved in the synthesis of bile acids, inflammation mediators and docosahexanoic acid (a modulator of neuronal function) (Islinger and Schrader, 2011).

Peroxisomes can form by growth and division from pre-existing peroxisomes or by *de novo* formation from the ER. Peroxissomal division is preceded by membrane elongation through a mechanism that involves the peroxin Pex11p. The final requires a dynamin-related protein with GTPase activity and associated adaptor proteins such as DLP1 and Fis1 and Mff in mammals. Remarkably, these components are shared with mitochondria. On the other hand, the de novo formation of peroxisomes from the ER is less understood but it seems to require a maturation process that involves the recruitment of new membrane and matrix proteins. The peroxissomal matrix proteins are synthesized in free ribosomes at the cytosol and are imported into the peroxisomes. An interesting feature is that peroxisomes are able to import fully folded or even oligomeric proteins. This import is dependent upon two peroxissomal targeting signals: PST1 and PST2; these signals are recognized by receptor peroxins (Pex5p

and Pex7p, respectively) and interact at a receptor docking site at the peroxissomal membrane (Schrader and Fahimi, 2008).

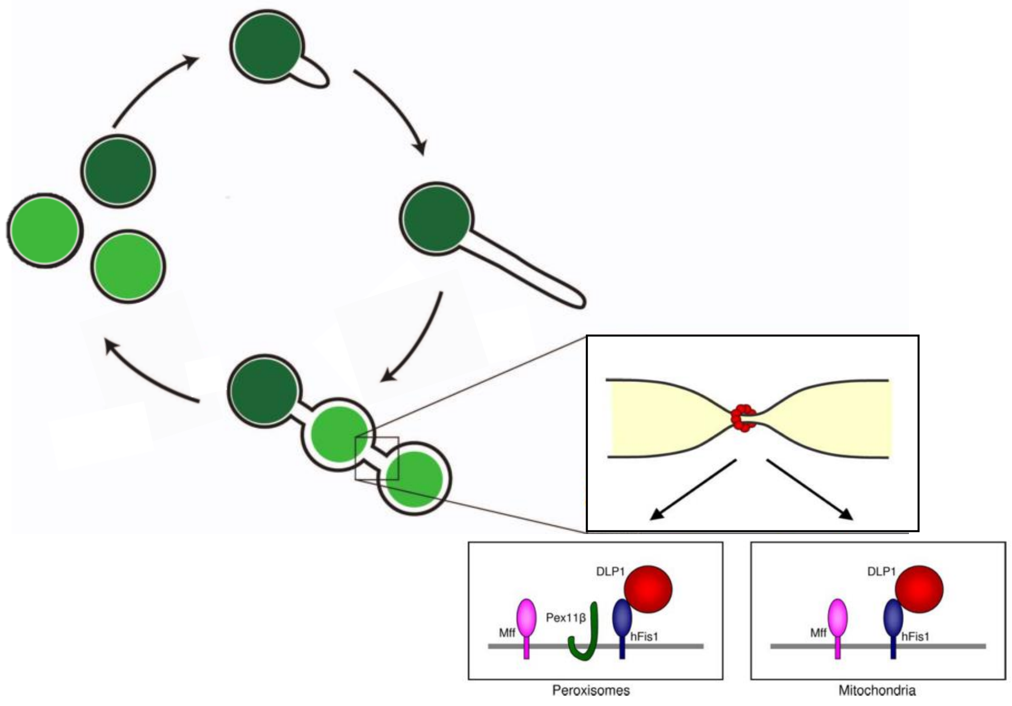

Figure 5 - Prototypical of peroxisome growth and division events.
The peroxisomal growth and division process begins with the formation of a small protuberance from a mature peroxisome that keeps extending to a larger one where new internal components are being synthesized. This extension starts acquiring constriction points from where the newly formed peroxisomes will separate and become individualized. At the same time, new proteins are imported to the forming peroxisomes. The final division occurs through the actions of DPL1 (which is responsible for the final division) and the membrane tail-anchored proteins Mff and Fis1 (the latter has been shown to interact with the elongation factor Pex11β), which recruit DLP1 to the constriction points. Mitochondria use a similar mechanism of growth and division. Adapted from: (Delille et al., 2009; Ribeiro et al., 2012)

It has become recently evident that peroxisomes are dynamic and interconnected organelles that actively contribute to signaling events, developmental decisions, ageing and pathogen defence (Islinger and Schrader, 2011). It was recently discovered that peroxisomes are involved in antiviral innate immunity through the membrane MAVS protein (Dixit et al., 2010)– greatly developed in chapter 4.

1.3 – Viruses: structural and taxonomic features; overview of the replication cycle

The **Virus'** universe is rich in diversity and they are known for their ability to infect all kinds of organisms, from mycoplasmas, bacteria and algae to all kinds of plants and animals. Viruses vary in their structure, genome, organization, expression and replication and transmission strategies (Adelberg, 2007).

The French bacteriologist Louis Pasteur was on the right path when we postulated that rabies was caused by a "living thing" smaller than bacteria and in 1884 was able to develop a vaccine against rabies. Pasteur also proposed the term virus (from the Latin "poison") to name this special group of infectious agents. In the 1890s, D. Ivanovski and M. Beijerinck demonstrated that a tobacco disease was caused by a virus. Latter, Friedrich Loeffler and Paul Frosch discovered that an animal virus caused the foot-and-mouth disease in cattle. In the following decades, a bigger picture of the physical, chemical and biological nature of viruses began to shape up and around 1950s virology had grown as a multi-layered discipline (Talaro, 2002).

1.3.1 - Viral Structure and Composition

The organizational plan of viruses is very simple, as they only comprise the necessary parts to invade and control host cells: an outer coating and a core with one or more DNA or RNA strands (Talaro, 2002).

All viruses have a protein **capsid** that surrounds the **nucleic acid** and central **core**. Together, capsid and nucleic acid are called as **nucleocapsid.** Some members of viral families have an additional coating named **envelope** that is nothing more than a modified part of membranes of the host. Viruses that only possess a nucleocapsid are called **naked viruses** and have different mechanisms than enveloped viruses of entering and exiting host cells. The virus particle is commonly designated as **virion** (Talaro, 2002).

The capsid is composed of identical subunits called **capsomers**. These capsomers self-assemble into the final capsid structure, and, depending on their shape, they can be arranged in two different forms: **helical** and **icosahedral** (Talaro, 2002).

Helical capsids are simple with rod-shaped capsomers that bind a series of hollow discs to form a structure that resembles a bracelet. These discs bind each other to form

a continuous helix in which the nucleic acids are coiled. The **icosahedral capsids** form a three dimensional structure (20 sides of equilateral triangles with 12 corners), with capsomers arranged in different ways: some build their capsid from a single type of capsid while others contain different types. Individual capsomers can have a ring or dome like shape, and the packaging of the nucleic acid occurs in the centre of the icosahedron, forming the nucleocapsid (Talaro, 2002). In spite of their icosahedral shape, most capsids have a spherical or cubical appearance (Adelberg, 2007).

Viruses only have one kind of nucleic acid, RNA or DNA – never both at the same time – that encodes the genetic information necessary for viral replication. Their genome can be single or double stranded, linear or circular, segmented or non-segmented, and the type of nucleic acid, their strandedness and size are the main features used to classify viruses into families (Adelberg, 2007).

A given number of virus families have an envelope in addition to the capsid in their structure. This lipid layer is acquired when the nucleocapsid buds through host membranes (organelle membranes or the cell membrane itself) during its maturation process. The phospholipidic content of the viral envelope is determined by the cell membrane type that is involved in the budding process. There are also glycosylated viral proteins protruding from the envelope that are exposed at the surface of the viral particle – designated **peplomers**. These glycoproteins are usually the ones involved in the attachment of the viral particle to specific host receptors – representing important viral antigens – and are also involved in the fusion of the envelope with the host cell membrane (Adelberg, 2007).

The main purpose of viral proteins is to facilitate the transfer of the nucleic acid from a host cell to another. In order to do so, they protect the viral genome against nuclease inactivation and provide structural symmetry to the viral particle. Some viruses possess enzymes such as RNA polymerase and reverse transcriptase, inside their virions that are essential to the beginning of the replication cycle when the virion enters the host (Adelberg, 2007).

1.3.2 - Classification and Taxonomy

The following features are used to classify and organize viruses into families:

1- Virion morphology, including size, shape, type of symmetry, presence or absence of peplomers and/or membranes

2- Viral genome properties such as the type of nucleic acid, genome size, strandedness (single stranded, double stranded), linearity or circularity, sense (positive, negative or ambisense), segments (presence, number and size), nucleotide sequence, G+C content, and the presence of special features (repetitive elements, isomeration, 5' terminal cap, 5' terminal linked protein, etc.)

3- Physical and chemical properties of the virion, including molecular weight, buoyant density, pH and heat stability, susceptibility to chemical and physical agents such as ether and detergents.

4- Viral proteins properties such as size, number, functional activities, amino acid sequence, modifications, etc.

5- Genome organization and mode of replication: genetic order, number and position of open reading frames, replication strategies and patterns of transcription and translation, cellular sites, etc.

6- Antigenic properties

7- Biological properties such as the host range, modes of transmission, vector relationships, pathogenicity, tissue tropism and pathology (Adelberg, 2007).

Taking into account these special features, it was established a taxonomy system where viruses are separated in large groups designated as **families**; the names of the families have the suffix –*viridae*. Within each family, there can be **subfamilies** (suffix – *virinae*) that comprise subgroups called **genera** that are classified based in physicochemical or serologic differences. **Genus** names have the suffix – *virus* and **Orders** (suffix – *virales*) can be used to group virus families (Adelberg, 2007).

The latest virus taxonomy release (July 2013) by the International Committee on Taxonomy of Viruses (ICTV) had organized viruses into 8 Orders: *Caudovirales, Herpesvirales, Ligamenvirales, Mononegavirales, Nidovirales, Picornavirales and Tymovirales* and another one for virus families without an assigned order and together

they comprise around 103 families, with ~40 subfamilies and countless genera and genus (International Committee on Taxonomy of Viruses, 2013).

Another virus classification system was proposed in 1971 by David Baltimore, which divides viruses into seven groups according to their genome type (RNA, DNA, single-stranded (ss), double-stranded (ds), positive or negative sense) and their method of replication (see *Figure 6*) (Baltimore, 1971; ViralZone, 2014).

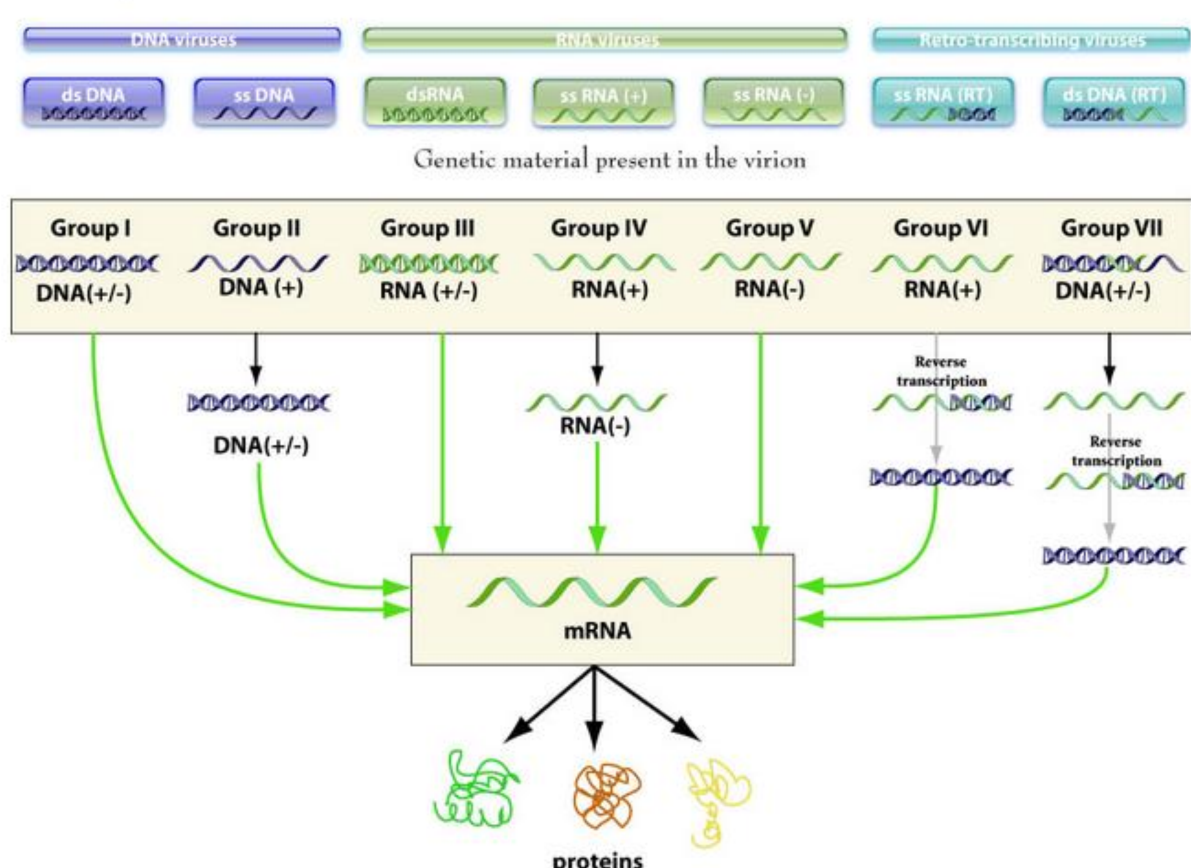

Figure 6 – The Baltimore Classification of viruses.
David Baltimore developed a virus classification system that groups viruses into families, depending on their type of genome. Group I encompasses double-stranded DNA viruses, which usually replicate within the nucleus of the host cell, requiring host polymerase in order to replicate their viral genomes. Group II covers all single-stranded DNA viruses, which also replicate within the nucleus. Group III includes double-stranded RNA viruses, that possess segmented genomes where each of the genes codes for a single protein. Group IV and V consist of single-stranded RNA viruses, with positive (group IV) or negative (group V) sense, which replicate in the cytoplasm (within their own capsids), not depending on host polymerases as much as DNA viruses do. Group VI comprises positive-sense, ssRNA viruses which require a DNA intermediate, using reverse transcriptases to convert the +ssRNA into DNA(Baltimore, 1971). Lastly, group VII covers dsDNA viruses, who are not considered DNA viruses (like in Group I viruses), but rather reverse transcribing viruses, as they replicate through a RNA intermediate (Temin, 1985).

Table 1 – Virus' classification according to family, virion size, type of symmetry, presence or absence of envelope, genome features and size, replication strategy and known viruses of each family or associated pathologies.

Adapted from: (Adelberg, 2007) *and* (ExPASy, 2014)*; images courtesy of* (ExPASy, 2014; The Science Picture Company, 2014; Zygote Media Group Inc., 2014)*; (+) – positive sense strand; (-) – negative sense strand; ss – single stranded; ds – double stranded; kb – kilobases; kbp – kilobase pairs; nm – nanometers*

	Virus Family	Virion Size (diameter)	Type of Symetry	Envelope	Genome Features	Genome Size	Replication Strategy	Viruses/Pathologies	
DNA Viruses	**Parvoviridae**	18-26 nm	icosahedral (32 capsomers)	no	linear; ssDNA	5.6 kb	nuclear replication in hosts undergoing active cell division	Parvovirus B19 - causes a childhood exanthem called "fifth disease"	Parvovirus B19
	Polyomaviridae	45 nm	icosahedral (72 capsomers)	no	circular; dsDNA	5 kbp	nuclear replication	JC virus (leucoencephalopathy), BK virus (mild respiratory infection), KI virus, WU virus	Murine Polyomavirus
	Papillomaviridae	55 nm	icosahedral (72 capsomers)	no	circular; dsDNA	8 kbp	nuclear replication	Human papillomaviruses - genital cancers	Human Papillomavirus 16
	Adenoviridae	70-90 nm	icosahedral (252 capsomers)	no	linear; dsDNA	26-45 kbp	nuclear replication	51 types; Adenovirus serotype 14 (severe respiratory infection)	Human Adenovirus
	Herpesviridae	150-200 nm	icosahedral (162 capsomers)	yes	linear; dsDNA	125-240 kbp	nuclear replication	herpes simplex type 1 (oral) and type 2 (genital), varicella-zoster, cytomegalovirus, Epstein-Barr	Herpes simplex

DNA Viruses	**Anelloviridae**	30-32 nm	icosahedral	no	circular; ssDNA	3,8 kb	nuclear replication	Alphatorquevirus; Betatorquevirus; Gamatorquevirus; Asymptomatic; may be associated with hepatitis, pulmunary diseases, myopathy and lupus	Anelloviriade structure
	Hepadnaviridae	40-48 nm	icosahedral	yes	circular; dsDNA	3,2 kb	nuclear and cytosolic replication	Hepatitis B virus; chronic hepatitis; can develop to liver cancer	Hepatitis B virus
	Poxviridae	220-450X140-260 nm	brick-like or ovoid structure	yes	linear; dsDNA	130-375kbp	exclusively cytosolic replication	Smallpox; Vaccinia; Molluscum contagiosum	Smallpox virus
RNA viruses	**Picornaviridae**	28-30 nm	icosahedral	no	+ssRNA	7.2-7.8 kb	cytosolic replication	Enteroviruses; Rhinoviruses; hepatoviruses (hepatitis A)	Human Rhinovirus
	Astroviridae	28-30 nm	icosahedral	no	linear; +ssRNA	6.4-7.4 kb	cytosolic replication	gastroenteritis	Astrovirus
	Caliciviridae	27-40 nm	icosahedral	no	+ssRNA	7.4-8.3 kb	cytosolic replication	Norwalk virus (acute gastroenteritis)	Norwalk virus

RNA viruses									
	Reoviridae	60-80 nm	icosahedral	no	linear; segmented; dsRNA	16-27 kbp	cytosolic replication	Rotaviruses that cause gastroentheritis	Rotavirus
	Togaviridae	65-70 nm	icosahedral	yes	linear; +ssRNA	9.7-11.8 kb	cytosolic replication	Rubella virus	Rubella virus
	Flaviviridae	40-60 nm	spherical or pleomorphic	yes	+ssRNA	9.5-12.5 kb	cytosolic replication	Dengue and Yellow Fever viruses	Dengue virus
	Arenaviridae	120 nm	pleomorphic	yes	circular; segmented; -ssRNA or ambisense	10-14 kb	cytosolic replication	Hemorrhagic fever syndromes; Guanarito virus, Junin virus, Lassa virus, Lujo virus, Machupo virus, Sabia virus	Lassa virus
	Coronaviridae	120-160 nm	spikes arranged in a fringe at the surface (like a solar corona)	yes	+ssRNA	27-32 kb	cytosolic replication	SARS (severe acute respiratory syndrome)	Coronavirus
	Retroviridae	80-110 nm	icosahedral	yes	2 copies of +ssRNA; linear	7-11 kb each copie	nuclear replication; reverse transcriptase	leukemia and sarcoma viruses; HIV	Human Immunodeficiency virus (HIV)

RNA Viruses	**Orthomyxoviridae**	80-120 nm	helical	yes	linear; segmented -ssRNA	10-13.6 kb	nuclear replication; hematoglutinin and neuraminidase activity	Influenza viruses	Swine Influenza virus (H1N1)
	Bunyaviridae	89-120 nm	spherical or pleomorphic	yes	triple-circular segment; -ssRNA or ambisense	11-19 kb	cytosolic replication	Hemorrhagic fevers and neuropathies; severe pulmonary syndrome	Orthobunyavirus structure
	Bornaviridae	80-125 nm	spherical	yes	linear; -ssRNA	8.5-10.5 kb	nuclear replication	Borna disease (horses)	Borna virus
	Rhabdoviridae	~78x180 nm	bullet-shaped	yes	linear; -ssRNA	13-16 kb	cytosolic and nuclear (plants) replication	Rabies virus, Vesicular stomatitis virus	Rabies virus
	Paramyxoviridae	150-300 nm	pleomorphic	yes	linear; -ssRNA	16-20 kb	hematoglutinin activity	mumps, measles, respiratory syncytial viruses	Mumps virus
	Filoviridae	~89X1000 nm	pleomorphic	yes	linear; -ssRNA	19 kb	cytosolic replication	Marbog and Ebola viruses	Ebola virus

RNA viruses	**Arboviridae**	ecological group; not a viral family; diverse chemical and physical properties; complex replication cycle --> use arthropods as vectors of infection to vertebrate hosts through mosquito/tick bite	
Other Viruses	**Viroids**	not adequate with typical definition of viruses; nucleic acids without protein coating; in plants are ssRNA; viroid RNA doesn't encode protein products; replication mechanism completely oblivious	
	Prions	absense of nucleic acid, only proteins - might not be a virus at all; hihgly resistant to heat, formaldahyde and UV light inactivation; enconded by a celular gene;cause prion disease - transmissible spongiform encephalopathies	Prion (Mad Cow disease)

1.3.3 - Viral Replication Cycle

Viruses can only multiply in living cells and the host must provide the energy and synthesis machinery and precursors for the synthesis of viral proteins and nucleic acids (Adelberg, 2007). Although this cycle is a continuous process, it helps to define the replication cycle into sequential events. The general phases of the animal viruses' cycle are as follows: Adsorption/Attachment → Penetration and Uncoating → Replication (viral genome expression and viral components synthesis) → Assembly/Morphogenesis →Release (*Figure 7*). The length of an entire replication cycle varies from 8 hours in polioviruses to 36 hours in herpesviruses (Adelberg, 2007; Talaro, 2002).

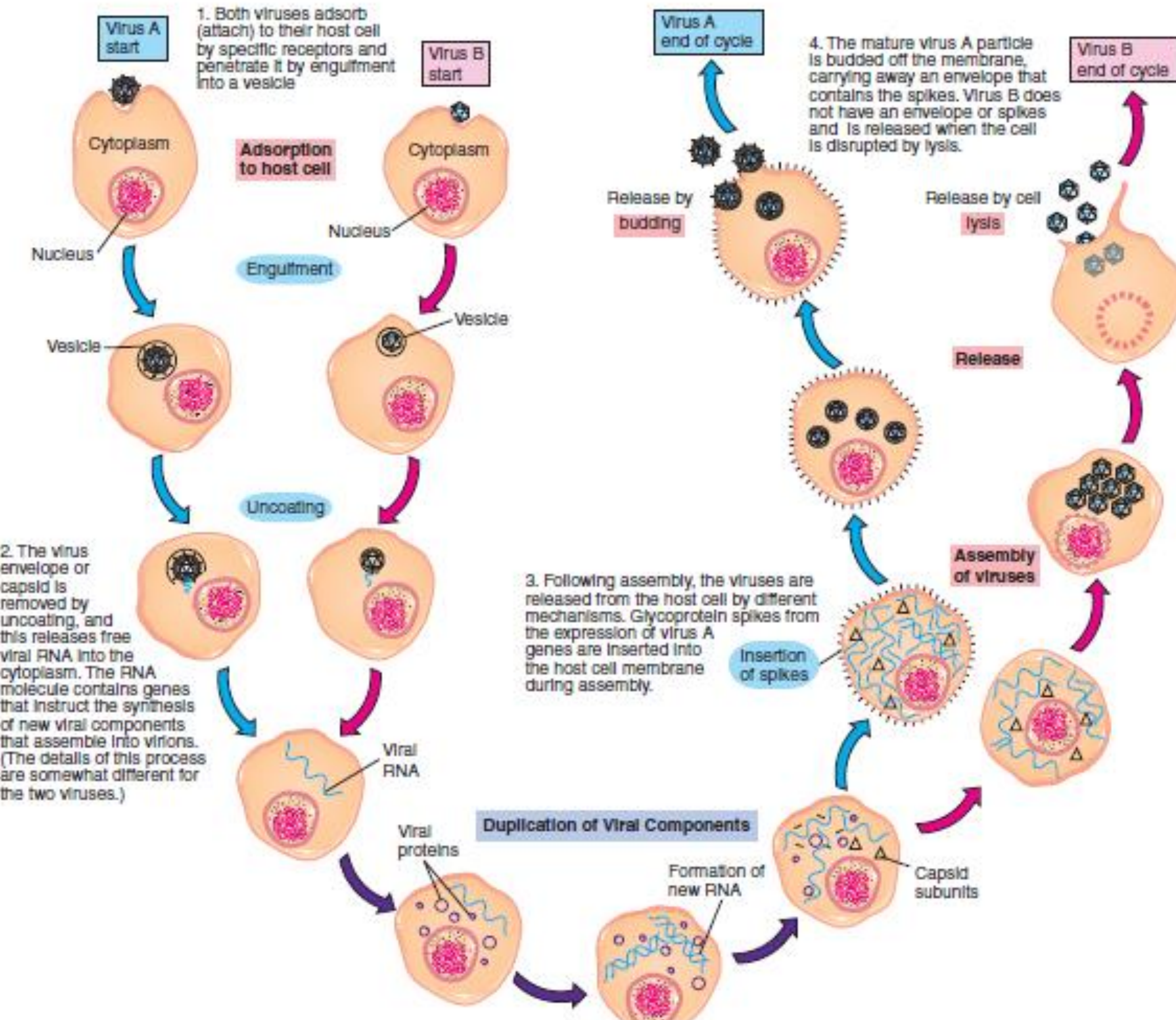

Figure 7 – General representation of a replicative cycle in animal viruses
Two types of viruses are compared: an enveloped and a naked virus. (Talaro, 2002)

1.3.3.1 - Adsorption, Penetration and Uncoating

The first step in viral infection is the **attachment** of a virion with a specific receptor at the surface of the host cell membrane. These receptors are usually glycoproteins, however, in certain cases viruses can bind to protein sequences or to oligosaccharides. The presence or absence of the receptors is a determinant for cell tropism and viral pathogenicity, since not all cells in a host organism express the necessary receptors. For example, HIV virus binds to CD4 receptor in immune cells while Epstein-Barr virus recognizes the CD21 receptors in B cells (Adelberg, 2007).

Upon binding to the cell surface, the virion is internalized – **penetration**. Some viruses use receptor-mediated endocytosis with uptake of the viral particles inside endosomes, while others (usually the enveloped viruses) penetrate directly their viral particles through the cell membrane with the interaction of a viral fusion protein with a secondary receptor (Adelberg, 2007).

The **uncoating** can occur simultaneously or right after penetration and it consists in the physical separation of the nucleic acid from the external virion structure. The viral genome can be released in the form of free nucleic acids or nucleocapsids that usually have polymerases (Adelberg, 2007).

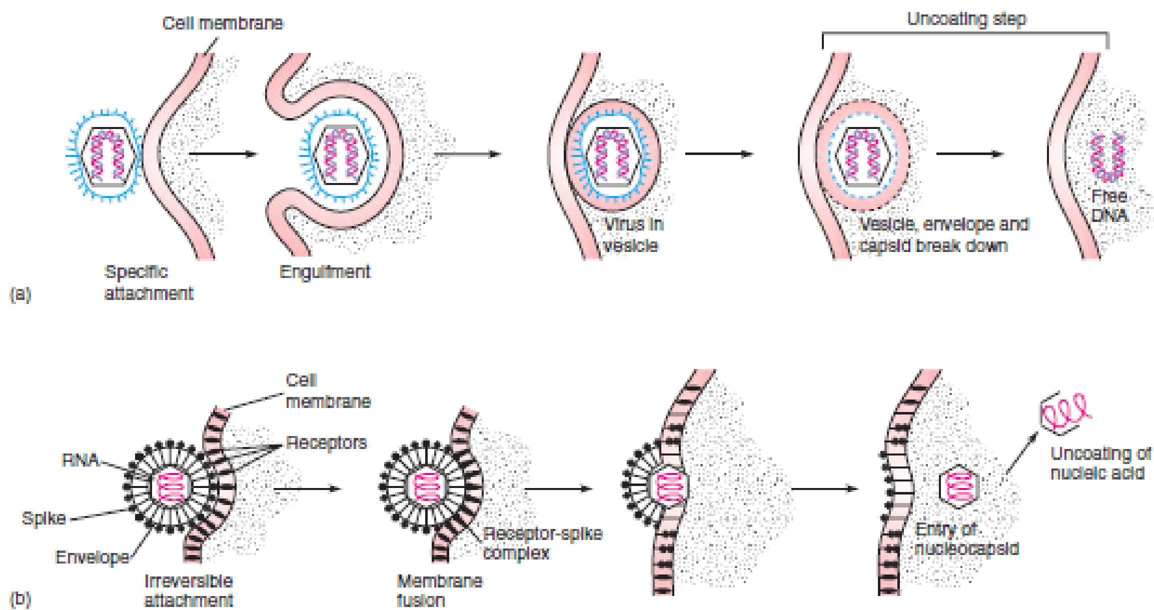

Figure 8 – Means of penetration of the host cell membrane by animal viruses.
(a) endocytosis and uncoating of a non-enveloped virus; (b) Fusion of the cell membrane with the viral envelope (mumps virus). (Talaro, 2002)

1.3.3.2 - Replication

Viral nucleic acids must be transcribed into mRNAs in order to the expression and duplication of the genetic information to be successful. Once this is achieved, viruses hijack cell components to translate these newly formed mRNAs. Different groups of viruses use different pathways to synthesize mRNAs depending on the structure of the nucleic acid (RNA or DNA), and some of them require RNA polymerases in order to synthesize their mRNAs (Adelberg, 2007).

During the course of viral replication, all virus-specific molecules are synthesized in a well-coordinated fashion; some are produced in an early stage of the infection while others only begin their synthesis later on. There's also a quantity control, since not all molecules are produced in the same quantity (Adelberg, 2007).

The intracellular sites where the different replication events take place also varies from group to group (*Table 1*) but, in general terms, viral proteins are synthesized in polyribosomes with virus-specific mRNAs in the cytosol; viral DNA is usually replicated within the nucleus while viral RNA is duplicated in the cytosol, with a few exceptions (Adelberg, 2007).

1.3.3.3 - Assembly and Release

The newly synthesized viral genomes, proteins and capsids all come together to form the **progeny viruses**. General rule, non-enveloped viruses accumulate inside the infected cells until they end up lysing and releasing the viral particles. Enveloped viruses mature through a **budding** mechanism. Specific envelope glycoproteins are inserted at the cell membrane so that nucleocapsids can bud through the membrane at these sites, and doing it, they acquire their envelope. Budding usually occurs at the cell membrane but it can happen in other cell membranes (i.e., Golgi apparatus, endoplasmic reticulum) (Adelberg, 2007).

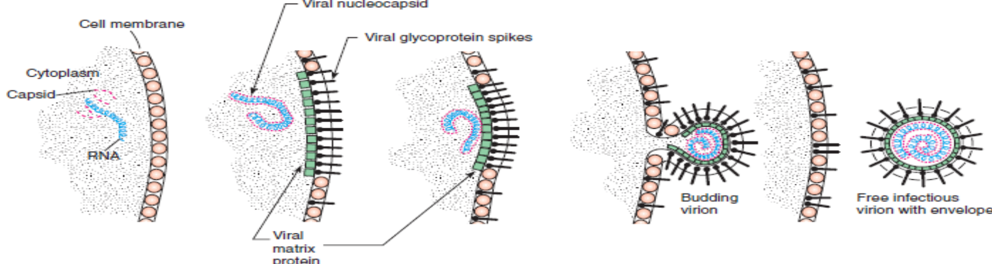

Figure 9 – Maturation of an enveloped virus with acquisition of envelope and spikes (parainfluenza virus).

(Talaro, 2002)

1.4 – Host defence mechanisms: Innate Immune response and Pattern Recognition Receptors

The defence systems have evolved to protect cells against the invasion of pathogenic microorganisms, being a system that is able to create a variety of cells and molecules that act together in a dynamic network in order to specifically recognize and eliminate a never ending variety of invaders. Functionally, the immune response can be divided into two phases: **recognition** and **response**. The recognition is extremely specific and it is able to distinguish subtle chemical differences that differentiate one pathogen from another. Besides, it is able to tell apart foreigner molecules from the ones of the own organism. Once the pathogen is recognized, the immune system recruits an array of cells and molecules in order to induce an effector response so that the invader organism can be neutralized and eliminated. Thus, the immune system is capable of converting the initial recognition event and processing it into effector responses, each response being highly specific to clear a particular type of pathogen. A later exposure to the same pathogen may induce a **memory response**, characterized by a faster and stronger immune reaction that acts in order to clear the pathogen and prevent the development of a disease state (Kindt et al., 2007).

The immune system is typically divided in two main components: the **innate immune response** that provides the first line of defence against infections (i.e.: barriers like the skin, mucous membranes, phagocytic cells, macrophages and neutrophils) and it is unspecific for any given pathogen; and the **adaptive immune response** that is highly specific, only takes place after the presentation of an antigenic challenge and possesses a “memory” property (the main agents are lymphocytes and antibodies). Since this second type of response takes some time to develop (approximately five to six days after the initial exposure to the pathogen), the innate response provides the first line of defence against the critical period, right after the exposure to the pathogen (Kindt et al., 2007).

The innate immune response comprises four types of defence barriers: anatomical, physiological, phagocytic and inflammatory (Kindt et al., 2007).

The physical and **anatomic barriers**, such as the skin and mucous surfaces/membranes, are the actual first line of defence against infections, since they are effective barriers against the entry of most microorganisms. For example, the skin

has sebaceous glands that produce oily secretions named sebum, which maintains the skin pH between 3 and 5, inhibiting the growth of most microorganisms. However, breaks in the structure of the skin such as scratches, wounds or abrasions or even the bite of insects might introduce pathogens into the lower layers of the skin and present a potential route of infection. On the other hand, the conjunctivae and food, respiratory and urogenital tracts are lined by mucous membranes with an external epithelial layer and a connective tissue layer underneath. Saliva, tears and mucous secretions have antibacterial or antiviral substances that act in order to wash away potential invaders. Mucous traps foreigner microorganisms and in the lower respiratory tract, the mucous membrane is covered by cilia (hair-like protrusions from the cell membrane of epithelial cells); the synchronized movement of cilia propels the pathogens trapped in the mucous outside of this tract (Kindt et al., 2007).

The **physiological barriers** include temperature, pH and soluble molecules associated with cells. The gastric acidity is an example of this type of barrier, since very few microorganisms can survive in such environment. Soluble factors such as lysozyme, interferons and complement proteins also contribute to this kind of innate barrier. Lysozyme is an hydrolytic enzyme found in mucous secretions and in tears and it is able to cleave the peptidoglycan layer of the bacterial wall. Interferons constitute a group of proteins produced by virus-infected cells that function to bind to adjacent cells and induce a generalized antiviral state. Several specific and unspecific mechanisms are able to activate complement proteins, render them able to damage the pathogen membranes either destroying them or facilitating their clearance. Collectins were also found to kill certain types of bacteria by direct breakdown of their lipid membranes or by aggregating bacteria to augment the susceptibility to phagocytosis (Kindt et al., 2007).

Another important defence mechanism is the ingestion of pathogens or extracellular materials through **phagocytosis**. Most of phagocytic events are taken by specialized cells such as blood monocytes, neutrophils or tissue macrophages (Kindt et al., 2007).

Tissue damage caused by wounds or pathogenic invaders induces a complex sequence of events known as **inflammatory response**. Certain microbial components can trigger an inflammatory response through interactions with cell surface receptors and the final result can be the recruitment of a specific immune response or the clearance of the invader by the components of innate immune response. The cardinal signs of inflammation – redness, swelling, heat, pain and sometimes loss of function – reflect the three main events of the inflammatory response (Kindt et al., 2007).

Vasodilatation of the surrounding capillaries takes place as the blood vessels that transport blood constrict, resulting in the ingurgitation of the capillary network (responsible for the redness and increase in tissue temperature). An increase of capillary permeability facilitates the influx of exudate and cells from the capillaries into the tissue and the accumulation of exudate contributes to the tissue swelling (edema). The migration of phagocytic cells to the capillaries walls (margination) is followed by the intake from the capillaries into the tissue (diapedesis) and finally the migration through the tissue to the site of infection (chemotaxis). As the phagocytes accumulate and begin their phagocytic activities, lytic enzymes are released and can damage the surrounding cells. The accumulation of dead cells, digested material and fluids forms the known pus (Kindt et al., 2007).

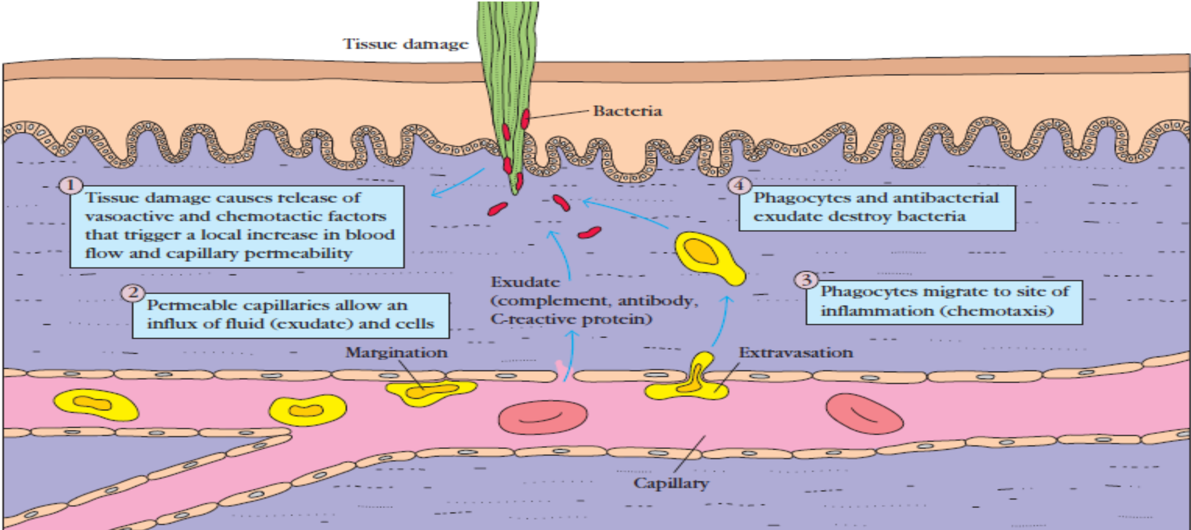

Figure 10 – The Inflammatory response.
The entry of a foreign body into the tissue causes tissue damage accompanied by the release of vasoactive and chemotactic factors. These factors induce increased blood flow to the affected area, increased capillary permeability and influx of white blood cells such as phagocytes and lymphocytes from the blood into the tissue. (Kindt et al., 2007)

Once the inflammatory response decreases and most of the debris are cleared by phagocytic cells, tissue repair and regeneration begin. Capillaries grow into fibrin of a blood clot and new cells of connective tissue (fibroblasts) replace fibrin and the clot dissolves. As the fibroblasts and capillaries accumulate, scar tissue is formed (Kindt et al., 2007).

1.4.1 – Pattern Recognition Receptors

In order to properly initiate an immune response against an infectious agent, the innate immune cells must have “something” that allows them to recognize certain microbial components patterns (commonly known as Pathogen-associated molecular patterns – **PAMPs**) which are inherent to the majority of invader pathogens. Otherwise they can recognize danger signals exposed at the surface of or released from damaged cells (Danger-associated molecular patterns – **DAMPs**). In fact there are cytosolic molecules and cell surface receptors with the ability of recognizing pathogen patterns, the so called, pattern-recognition receptors (PRRs). These recognized patterns include combinations of sugars, certain proteins, particular molecules with lipid content and some nucleic acid motifs. The ability of PRRs to distinguish self and nonself components is remarkable, since the targeted molecular pattern is only produced by the pathogen and never by the host (Kindt et al., 2007).

There are multiple families of PRRs, the best characterized including those associated with the cell membrane such as the **Toll-like receptors (TLRs)** and **C-type lectin receptors (CLRs)** and cytosolic receptors such as the **NOD-like** and **RIG-I-like receptors (NLRs** and **RLRs**, respectively). In general terms, the recognition of ligands by PRRs triggers signal transduction pathways which results in the expression of pro-inflammatory cytokines, chemokines and antiviral molecules (interferons); with the exception of NLRs that lead to the formation of multiprotein complexes named *inflammasomes* that serve as cleavage and activation platforms for caspase-1. Caspase-1 promotes maturation and secretion of IL-1β (interleukine-1β) and IL-18, which amplifies even further the pro-inflammatory response [reviewed at (Koyama et al., 2008; Wilkins and Gale, 2010)].

1.4.1.1 – Toll-like receptors

Toll-like receptors are a transmembrane family of PRRs that are expressed in a variety of immune and non-immune cells, including monocytes, macrophages, dendritic cells, neutrophils, B and T cells, fibroblasts, endothelial and epithelial cells. TLRs initiate the immune response after the recognition of PAMPs presented in microbial molecules or endogenous DAMPs released by damaged cells. There are ten functional TLRs in humans: TLR 1, 2, 4, 5, 6 and 10 are expressed at the cell surface and mainly recognize membrane or wall components of pathogens, while TLR 3, 7, 8 and 9 are expressed at membranes of endolysosomal compartments and recognize nucleic

acids. TLRs have a variable number of ligand-sensing, leucine-rich repeats at their N-terminal and a cytosolic domain Toll-interleukin-1 receptor (TIR). TIR domain mediates interactions between TLRs and adaptor proteins involved in the regulation of the downstream signaling such as MyD88 (Myeloid differentiation primary response gene 88), TRIF (TIR-domain-containing adapter-inducing interferon-β), TRAM (Toll-receptor-associated molecule) and TIRAP (toll-interleukin 1 receptor (TIR) domain-containing adaptor protein; also named MAL [MyD88 adapter-like]). The signaling pathways downstream promote the expression of pro-inflammatory cytokines, chemokines and type I and III interferons (Kawai and Akira, 2011).

1.4.1.2 – C-type lectin receptors

C-type lectin receptors (CLRs) are a diverse family of soluble and transmembrane proteins that have one or more C-type lectin-like domains (CTLDs) and are particularly important in antifungal immunity. Most CLRs that function as PRRs belong to the Dectin-1 or the Dectin-2 subgroups. Members of these groups are transmembrane proteins expressed mainly in monocytes, macrophages and dendritic cells that recognize fucose, mannose or glycan carbohydrate structures. Dectin-1 and dectin-2 are the best characterized CLRs that function as PRRs and both have been showing to promote NF-kB canonical signaling through the activation of SYK (spleen tyrosine kinase) and a multiprotein complex of CARD9, Bcl-10 and MALT1; they also activate NFAT and AP-1, and dectin-1 also regulates NF-kB activity through the non-canonical NF-kB pathway and through Raf-1-mediated phosphorylation and acetylation. As a result, the signaling pathways initiated by either of these two CLRs can control the expression of numerous cytokines that direct the innate and adaptive immune response. Signaling pathways activated by CLRs can also directly regulate or modulate TLR signaling; upon activation, DCIR (Dendritic Cell Immunoreceptor) and MICL (Myeloid C-type lectin-like receptor) recruit the phosphatases SH2-domain-containing protein tyrosine phosphatase 1 (SHP-1) and SHP-2 and inhibit TLR8 and TLR9 (Hoving et al., 2014).

1.4.1.3 – NOD-like receptors

The **Nucleotide-binding oligomerization domain (NOD)-like receptors** (NLRs) are cytosolic receptors that provide a second line of defence against invader pathogens. The NLR family comprises 22 proteins that are divided in subfamilies

(NLRA, NLRB, NLRC, NLRP e NLRX) based on their protein interacting N-terminal domains; additionally, all NLRs have a nucleotide-binding/oligomerization domain and a variable number of ligand-sensing, leucine-rich repeats at their C-terminals. NOD1 and NOD2 are the best characterized NLRs that belong to the NLRC family, recognizing bacterial peptidoglycan components. Upon activation, NOD1 and NOD2 homodimerize and recruit signaling molecules leading to the pro-inflammatory cytokine-dependent NF-kB/AP-1 expression and type I interferon expression, dependent of IRF-3/IRF-7. Other NLRs are activated by a different array of pathogens or endogenous danger signals and oligomerize in order to form multiprotein complexes inflammasomes. The oligomerization into inflammasomes induces the cleavage and activation of caspase-1 that promotes the processing and secretion of IL-1β and IL-18 and can induce cell-death known as pyroptosis (Kanneganti, 2010).

1.4.1.4 – RIG-I-like receptors

The **RIG-I-like receptors** (RLRs) are a family of cytosolic sensors for viral RNAs that include the retinoic acid-inducible gene-I (RIG-I), the melanoma differentiation-associated 5 (MDA5) and the laboratory of genetics and physiology 2 (LGP2). These RLRs are composed of a DexD/H box RNA helicase domain, a C-terminal repressor domain and two caspase recruitment domains at their N-terminal. The LGP2 doesn’t have the CARD domain as the other two RLRs, consisting of a RNA helicase domain and a repressor terminal. RIG-I and MDA5 function as cytoplasmic sensors of RNA to induce the IFN-I production in non-immune cells (Loo and Gale, 2011).

The two best characterized RIG-I-Like Receptors (RLRs), RIG-I and MDA5, are ubiquitously expressed in the cytosol of several cell types, and are able to recognize structurally distinctive RNA species that reach the cytosol of the host cell, by infection or by means of transfection (Dixit and Kagan, 2013).

The Retinoic acid-inducible gene-I (RIG-I) was first identified as an induced gene in promyelocytic leukaemia cells after treatment with retinoic acid (Sun, 1997), while the melanoma differentiation associated gene 5 (MDA5) was first identified in a differentiation-induction subtraction hybridization screen, designed to define genes regulated upon induction of differentiation, in human HO-1 melanoma cells (Jiang and Fisher, 1993).

Evidences suggest that RIG-I resides in membrane ruffles on non-polarized epithelial cells, where it associates with the F-actin cytoskeleton Alternatively, MDA5

localizes in the cytosol, with no visible co-localization with F-actin. Further observations have showed that RIG-I is capable of inducing cell migration within its association with F-actin and it also localizes at apical junction complexes in polarized epithelial or endothelial cells. The biological implications of the RIG-I – F-actin interaction are significant: it appears that depolymerisation of actin results in redistribution of RIG-I and subsequent activation of IRF3, NF-kB, and the IFN-β promoter activity (Mukherjee et al., 2009).

Regarding its functions upon viral infection, RIG-I ligands encompass RNA molecules with two special features: (1) they possess a 5' triphosphate (Hornung et al., 2006) and (2) 5' base pairing due to secondary RNA structures, such as hairpin conformations or panhandle structures (Schlee et al., 2009; Schmidt and Schwerd, 2009).

Studies targeting the characterization of molecular features of RIG-I ligands are largely based on *in vitro* transcripts [reviewed in (Schlee and Hartmann, 2010)]. *In vitro* RNA transcription by all the known RNA polymerases leaves a triphosphate at the 5' terminal (pppRNA). Transfection of pppRNA in monocytes resulted in a robust secretion of IFN-α, while RNA without the triphosphate didn't (Hornung et al., 2006). However, the 5' triphosphate structure is not enough on its own to target a single stranded RNA (ssRNA) as nonself RNA and render it immunogenic – since studies revealed that 5' triphosphate ssRNA did not activated the RIG-I signaling (Schlee et al., 2009). Reverse cloning and sequencing of this ssRNA revealed the presence of sequences generated by self-coding intramolecular 3' extensions that lead to a blunt-ended RNA with complementary 5'and 3' sequences (Schlee et al., 2009). Thus, these aberrant products of *in vitro* transcription are responsible for the immune-stimulatory properties of such preparations (Schlee et al., 2009). Alternatively to the 5´ base pairing, the composition of the RNA sequence may as well contribute to the stimulatory potential of pppRNA (Saito et al., 2008; Uzri and Gehrke, 2009). For example, the genomic ssRNA of Hepatitis C virus (HCV) is characterized by poly-uridine motifs with interspaced C nucleotides (known as poly-U/UC motifs), as well as a 5' triphosphate (Saito et al., 2008; Uzri and Gehrke, 2009). Deletion of the poly-U/UC motifs abrogated the stimulatory activity of HCV (Uzri and Gehrke, 2009). Thus, both, the panhandle structures and poly-U/UC might serve as secondary PAMPs to pppRNA (Saito et al., 2008; Uzri and Gehrke, 2009).

So far, the understanding of the molecular nature of MDA5 ligands is very poor. The common agonist for MDA5 is poly I:C, a synthetic molecule without a 5' triphosphate

generated by annealing of poly-iosine strands to poly-citidine strands of variable sizes (Gitlin et al., 2006). Size fractionation of poly I:C revealed that MDA5 responds to high molecular weight poly I:C, while poly I:C with less than 1000bp acts as an agonist for RIG-I (Kato et al., 2008). *In vitro* assays using recombinant RIG-I and MDA5 showed that short and long poly I:C induce ATPase activity for RIG-I and MDA5, respectively (Kato et al., 2008). Consistent with this data, short dsRNA species (1.2-1.4 kbp) of reovirus genome selectively activated RIG-I, while longer dsRNA (~3.4 kbp) was able to activate MDA5 (Kato et al., 2008). These results strongly suggest that RIG-I and MDA5 discriminate short and long dsRNA; however, the underlying mechanism of the nucleotide length detection is yet to be discovered (Kato et al., 2008).

All three highly related proteins that constitute the RLRs family (RIG-I, MDA5 and LGP2) are presented at low levels in resting cells and its expression is strongly induced by type I interferons, creating a feed-forward feedback loop for a sustained antiviral response [reviewed at (Dixit and Kagan, 2013)].

RIG-I and MDA5 are DexD/H-box RNA helicases comprising two caspase activation and recruitment domains (CARDs) in their N-terminal – which are in fact the effector domains, responsible for signal transduction – while LGP2 lacks CARDs (Saito et al., 2007; Yoneyama et al., 2005) and reviewed at (Takeuchi and Akira, 2008; Yoneyama and Fujita, 2008). RIG-I and MDA5 share ~25% homology in their CARD regions and 40% in their helicase domain (Yoneyama and Fujita, 2008); however, it was not well understood how nonself RNA was physiologically recognized, since none of these helicases has a RNA-binding domain.

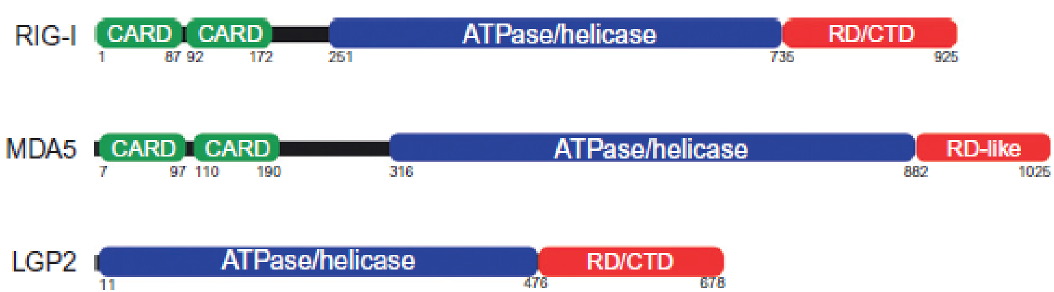

Figure 11 – Representation of the domains structures of RLRs and MAVS.
Schematic representation of the domain features of the three RLRs. RIG-I and MDA5 hold two CARD domains while LGP2 lacks CARD domains at the N-terminus. All three RLRs contain an ATPase/helicase domain followed by the repressor domain (RD) at the C-terminus (CTD) – MDA5 RD-like domain doesn't participate in auto-regulation. Adapted from: (Dixit and Kagan, 2013)

RIG-I and LGP2 also have a repressor domain (RD, aa. 723-925), localized at the C-terminal domain (CTD) (Saito et al., 2007). The repressor domain is capable of interaction with the CARD and helicase (helicase linker region, aa. 420-627) domains, and overexpression of the repressor domain blocks the immediate RIG-I signalling (Saito et al., 2007). Thus, it was proposed an auto-repression model, in which the CARDs are masked through intramolecular interactions, mediated by the RD (Saito et al., 2007).

The crystallographic structure of the repression domain reveals a zinc-binding domain, coordinated by four cysteines, also conserved in MDA5 and LGP2 (Cui et al., 2008). Mutation studies show that this zinc-coordination site is a key structural motif, essential for RIG-I signalling; however, further studies are required in this matter (Cui et al., 2008).

The C-terminal domain was recently described to partially overlap with the repressor domain, and the atomic structure of the former was determined by x-ray crystallography (Cui et al., 2008) and nuclear magnetic resonance (NMR) (Takahasi et al., 2008). One side of CTD exhibits a large cleft with positive surface charges and, the opposite side contains acidic patches (Takahasi et al., 2008). Adding dsRNA or 5'pppRNA specifically titrates the NMR signal, suggesting that this cleft is, in fact, the RNA recognition surface (Takahasi et al., 2008). Consistent with this observation, mutagenesis at the cleft reduced the RNA binding and the signalling ability of RIG-I (Takahasi et al., 2008). Additionally, *Cui et al.* demonstrated, through filtration-gel analysis, that RIG-I CTD recognizes 5'pppRNA as a dimer, so, it is probable that one dsRNA molecule can simultaneously bind multiple CTD RIG-I molecules (Cui et al., 2008).

Functional studies suggest that CTD detains two distinct functions: the RNA recognition and signal repression. Mutagenesis in the concave cleft inactivated RNA recognition, but none of the mutations led to a permanently active RIG-I, suggesting that the RNA recognition and the RNA repression surfaces don't overlap (Takahasi et al., 2008). Furthermore, ATP binding or its hydrolysis is not required to RNA recognition, suggesting a model of an inactive RIG-I, in which the repressor domain mediates a closed structure through intramolecular interactions, while the RNA-binding domain remains available (Cui et al., 2008; Saito et al., 2007; Takahasi et al., 2008). When a virus releases its dsRNA or 5'pppRNA, these PAMPs bind to the concave surface of the RNA-recognition site, inducing conformational changes in the presence of ATP, and, consequently, the CARDs are exposed (Takahasi et al., 2008). The freed

CARD domains form complexes with other molecules of RIG-I or with the protein adaptor MAVS (Mitochondrial AntiViral Signalling adaptor) for the progression of the downstream signal transduction (Cui et al., 2008; Takahasi et al., 2008).

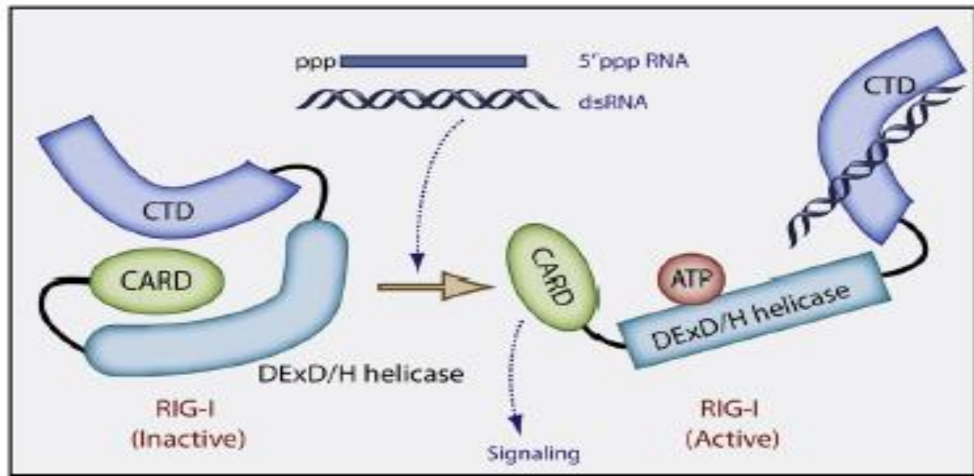

Figure 12 – Model of RIG-I activation by nonself RNA.
In an inactive state, the RIG-I CTD overlaps with the repressor domain and interacts with the helicase domain and the CARD in the absence of its ligand. When viruses produce dsRNA or 5'ppp-RNA, these nonself RNAs bind to the RNA-recognition cleft on the CTD and induce conformational changes in the presence of ATP, resulting in the exposure of the CARD. The released CARD becomes able to form complexes with either other RIG-I molecules or downstream adaptor MAVS to transduce biological signals (Yoneyama and Fujita, 2008).

Unlike RIG-I and MDA5, the LGP2 role in antiviral immunity is less clear. LGP2 lacks the CARD domain.(Rothenfusser et al., 2005; Yoneyama et al., 2005) Deployed of a signalling domain, LGP2 was proposed as a negative regulator of RLR signalling, since the overexpression of LGP2, in fact, does not activate induction of IFN-β.(Rothenfusser et al., 2005) *In vivo* experiments with different lines of LGP2-defficient mice contradicted the previous data generated by *in vitro* studies, implicating LGP2 as a positive regulator (Satoh et al., 2010). In the absence of LGP2, the viral RNA responses through RIG-I and, particularly, through MDA5 were compromised while responses to synthetic ligands weren't affected (Satoh et al., 2010). Presumably, LGP2 facilitates the binding of viral RNA – potentially through protein complexes – to its receptor, while the affinity of RIG-I and MDA5 is strong enough to bind naked synthetic agonists (not bound to LGP2) (Satoh et al., 2010; Venkataraman et al., 2007). Structural analysis of the binding interface between RNA and the CTD supports this model, since it predicts a weaker affinity of MDA5 than RIG-I to its ligand (Takahasi et al., 2009).

Besides confirming the role of LGP2 as a positive regulator (although not essential to RLR signaling), a recent study implicated LGP2 as an intrinsic regulator of the survival of $CD8^{+}T$ virus-specific cells (Suthar et al., 2012). CD8+T cells are crucial to control the pathology of the West Nile virus in the brain. LGP2-defficient mice presented a higher viral burden and a significant reduction of West Nile virus-specific $CD8^{+}T$ cells, leading to a higher mortality rate when compared to wild-type animals (Suthar et al., 2012). In spite of these findings, further clarification is still necessary to establish the role of LGP2 in RLR signaling pathways.

1.4.1.5 – Cytosolic DNA receptors

While RLRs focus their actions against viral RNAs, very recently, other molecules have been found to act as cytosolic receptors capable of direct recognition of viral DNA. Among them, DAI (DNA-dependent activator of IFN-regulatory factor) was the first DNA cytosolic sensor described to induce type I IFN in response to dsDNA (Takaoka et al., 2007). Several groups identified AIM2 (absent in melanoma 2) as a cytosolic dsDNA sensor, whose activation promotes the assembly of an inflammasome (Bürckstümmer et al., 2009; Fernandes-Alnemri et al., 2009; Hornung et al., 2009). A third cytosolic dsDNA sensor, LRRFIP1 (Leucine-rich repeat flightless-interacting protein 1) can recognize AT-rich B-form dsDNA as well as GC-rich Z-form dsDNA. *Yang et al.* demonstrated that LRRFIP1 triggers IFN-β production in a β-catenin-dependent manner (β-catenin binds to the C-terminal domain of IRF3, inducing an increase in IFN-β expression) (Yang et al., 2010). Despite the discovery of these cytosolic DNA sensors and their ligands, their precise role in antiviral innate immunity remains elusive.

Since a single pathogen can activate multiple PRRs, crosstalk between the different receptors might have a role in enhancing or inhibiting immune responses. Thus, the regulation of the PRR signaling is required in order to clear infectious pathogens and, at the same time, preventing an abnormal and excessive PRR activation that could lead to the development of auto-immune or inflammatory disorders.

1.5 – Cellular responses to viral infections: Interferons (IFNs) and Interferon-stimulated genes (ISGs)

1.5.1 - Interferons

There is no definitive cure to viral infections and, most of the times, their outcome relies entirely on the ability of the immune system to recognize, restrain and eliminate the virus. Based on current evidences, it seems that the immune system employs essentially the same type of mechanism against the endless array of viruses. This mechanism relies on the actions of **interferons (IFNs)**. This molecules are produced in infected cells and are capable of "warning" the neighbouring cells about the presence of a viral infection in course, so they can be better "prepared" to fight the infection (or even commit suicide through apoptosis), thus preventing the spread of the virus. To date, the key aspect for the establishment of an effective immune response relies on a robust production and secretion of IFNs in response to a given infection (Meager, 2006).

The interferon system is considered unspecific since several stimuli can induce the production of the same type of IFNs and the same type of IFNs inhibits several kinds of viruses. On the other hand, interferon can also possess very specific actions depending on the animal species in which they are induced (e.g.: IFNs protect human and monkey cells but not chicken cells) (De Andrea et al., 2002).

Based on their amino-acidic sequence, their receptor specificity, chromosomal location, structure and physicochemical properties, interferons are grouped in three distinct classes, namely, **type I** , **type II** and **type III** (Meager, 2006).

The mammalian type I interferons include IFN-α (alpha), IFN-β (beta), IFN-κ (kappa), IFN-δ (delta), IFN-ε (epsilon), IFN-τ (tau), IFN-ω (omega), and IFN-ζ (zeta, also known as limitin). This repertoire of interferons is thought to signal through the surface receptor complex **IFNAR** that consists in two transmembrane chains IFNAR-1 and IFNAR-2. However, not all type I interferons are expressed in humans; thus, the focus will be only upon IFN-α and -β, the first to be discovered and best characterized interferons (Meager, 2006).

IFN-α is encoded in a cluster of genes on chromosome 9 in humans. It is a protein family that comprises around 13 subtypes that share 76-99% of their amino acid

identity, whose structure comprises 166 amino acids with no glycolisation sites and a stable pH – with the exception of IFN-α2a that has a deletion on position 44. The different subtypes are produced differentially, depending on the inducer or producing cell. They are induced in macrophages and lymphocytes under the stimulation by tumour or virus-infected cells as well as prokaryotic cells and mitogens (De Andrea et al., 2002; Meager, 2006).

Unlike IFN-α, **IFN-β** is encoded by a single gene, also on chromosome 9 in humans. IFN-β proteins have a 166 amino acid sequence, sharing 40-50% of homology with IFN-α. They are expressed during myeloid differentiation and, as for IFN-α, in response to a given infection, particularly to Gram-negative bacterial infections (interestingly bacterial LPS doesn't induce IFN-α) (Meager, 2006).

Interferon-γ belongs to the type II class of interferons and is commonly known as the "immune" interferon. It is encoded on chromosome 12 and it is produced mainly in sensitized lymphocytes with the aid of macrophages and only under the action of mitogens. The IFN-γ protein has 166 amino acids with 23 of them representing a hydrophobic signaling sequence, with no analogy with IFN-α and –β (De Andrea et al., 2002; Meager, 2006).

Until 2003, the knowledge of immune responses to viruses was focused on type I and type II interferons; however, a new class of IFNs has been identified, the **type III interferons**. They comprise a family of proteins capable of inducing antiviral protection, designated by IFN-λ1, -λ2 and –λ3, alternatively, interleukins 29, -28A and -28B. Despite using a different receptor complex than type I IFNs, the main outcome is basically the same, resulting in the activation of Janus kinase (JAK)–Signal transducers and activators of transcription (STAT) signal transduction events, including the formation of IFN-stimulated gene factor 3 (ISGF3). A special feature of this class is that their actions are mainly limited to epithelial-like cells, providing antiviral protections only to certain tissues and organs (Meager, 2006).

In spite of interferons being acknowledged by their potent antiviral action, they were also found to affect other vital cells and body functions. They can increase cell death in granulocytes, macrophages, natural killer and cytotoxic lymphocytes and interfere with the humoral immune response and the antigen/receptor expression. They can also lyse or inhibit the growth of certain cells, influence the differentiation and hormonal functions of epinephrine and adrenocorticotropin (ACTH). Interestingly, the effect of these modulations can also influence the outcome of viral infections (Albrecht et al., 1996).

1.5.2 – Interferon-stimulated genes

The interferon system is famous for its ability to block viral replication and their effects are mediated by gene products whose expression is highly induced by interferons (Sen and Sarkar, 2007). These **Interferon-stimulated genes (ISGs)** were first discovered over 25 years ago, and, depending on cell type, interferon dosage, time of treatment, several microarray studies identified 50 to 1000 ISGs; In spite of these big numbers, only a small amount of action mechanisms of the ISG effector proteins were so far revealed. Collectively, ISGs aim at almost all steps of viral replication and present combinatory antiviral effects, acting synergistically to provide a powerful antiviral response. Some ISGs can even re-enforce antiviral effects inducing additional IFNs or ISGs (Schoggins and Rice, 2011). – refer to CHAPTER 2.4 for individual ISG action mechanisms.

2 – Mitochondria-dependent Antiviral Signalling – via the MAVS adaptor

2.1 – Overview of the mitochondria-dependent antiviral pathway

Mitochondria have been shown to be the key location for the signalling pathways initiated by RLRs. In fact, the mitochondrial antiviral signalling adaptor (MAVS), primarily localized in the mitochondrial outer membrane, has been characterized as a RIG-I binding protein (Ohta and Nishiyama, 2011).

Upon RNA virus infection, the RIG-I helicase domain senses RNA-specific structures, inducing conformational changes and exposure of CARD domains, which are then submitted to polyubiquitination by TRIM25 at Lys172 (Gack et al., 2007). This conformational change mediates the dimerization of RIG-I through CARD interactions and subsequent binding to MAVS, which, in turn, dimerizes to generate a signalling scaffold (Gack et al., 2007). The RIG-I – MAVS interaction leads to the recruitment of members of the TRAF (Tumour Necrosis Factor (TNF) Receptor-Associated Factor) adaptor family, resulting in the parting of the signalling pathway in two: the antiviral IFN response, mediated by TRAF3, or the inflammatory response, mediated by TRAF2 and TRAF6 (*Figure 13*) (Saha et al., 2006; Seth et al., 2005; Xu et al., 2005).

The type I IFN induction is mediated by an initial complex, consisting of TRAF3, NEMO (NF-κB Essential Modulator, also known as IKK-γ [Inhibitor of κB kinase, gamma subunit]) and TANK (TRAF family member-associated NF-kB activator). This complex controls the kinase activity of TBK1 (TANK-binding kinase 1) and IKKε (Inhibitor of κB kinase, epsilon subunit), which, specifically, phosphorylates the interferon regulatory factors IRF3 and IRF7, leading to their dimerization, nuclear translocation, and transcriptional activation of type I IFN genes (Belgnaoui et al., 2011).

On the other hand, TRAF2 and TRAF6, in cooperation with RIP1 (Receptor-Interacting Protein 1), activate the canonical kinase complex – IKKα, IKKβ and NEMO – resulting in the phosphorylation of IκBα at its Ser32/36 residues, leading to the ubiquitin-dependent proteasomal degradation of IκBα. IκBα is a regulatory protein that inhibits NF-kB by complexing with, and trapping it in the cytoplasm. The release of DNA subunits of NF-kB results in the nuclear activation of specific NF-kB target genes which regulate inflammatory responses (Belgnaoui et al., 2011).

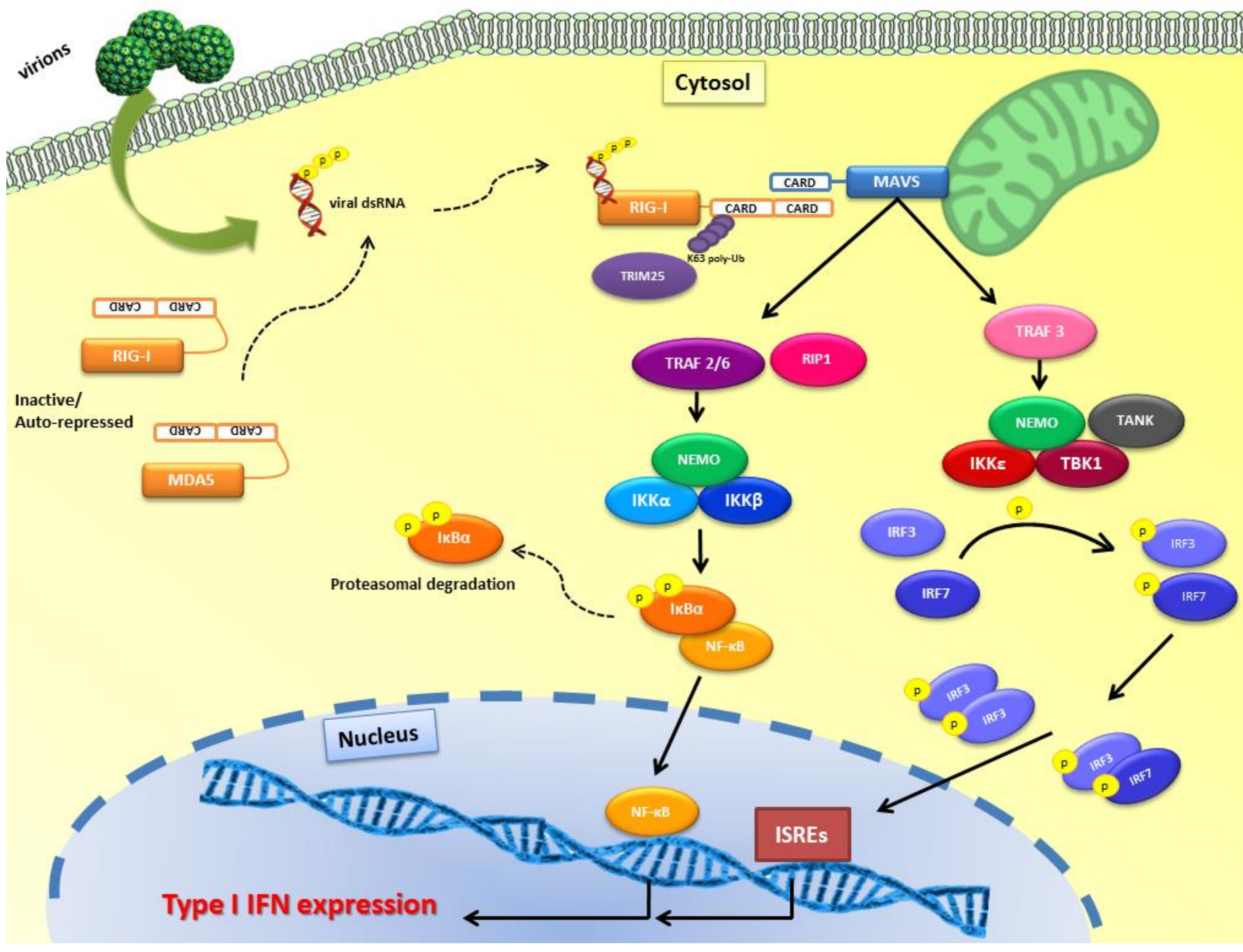

Figure 13 – Schematic representation of the RIG-I signalling pathway.

Upon detection of viral RNA with specific structure features (short dsRNA or 5'-pppRNA) RIG-I is subject of protein unfolding (exposing the CARD domains), CARD ubiquitination by TRIM25 and interaction with MAVS that is bound to the mitochondrial outer membrane through its transmembrane domain. MAVS dimerizes (forming prion-like aggregates at the surface of mitochondria) and recruits adaptor proteins which are responsible for the activation of the transcription factors NF-kB, IRF3 and/or IRF7. The NF-kB branch of this pathway is induced via the recruitment of TRAF2/6 and RIP1, which triggers the activation of the IKK complex (NEMO/IKKα/IKKβ) that in turn is responsible for the phosphorylation of the NF-kB inhibitor IκBα, causing its proteasomal degradation. Phosphorylation of this repressor releases NF-kB, promoting its nuclear translocation. The IFN branch of the RIG-I pathway is carried out by the interaction of TRAF3 with MAVS, which leads to the recruitment of the TANK/NEMO/IKKε/TBK1 complex, which is accountable for the phosphorylation, dimerization and nuclear translocation of IRF3 and/or IRF7. IRF3 and IRF7 dimers bind to ISREs (Interferon-Stimulated Response Elements) promoters to ultimately induce the expression of IFN-stimulated genes. Text adapted from: (Belgnaoui et al., 2011)

Both, IRFs and NF-kB bind to the IFN-β promoter in a temporary coordinated fashion to carry on its transcription. In turn, the secreted IFN-β binds to and activates the type I IFN receptor in an autocrine or paracrine manner. The receptor-ligand interaction induces the activation of the JAK-STAT pathway and formation of ISGF3 (IFN-stimulated gene factor 3), which translocates to the nucleus and induces the transcription of hundreds of ISGs (Interferon Stimulated Genes) involved in the generation of an antiviral state (Belgnaoui et al., 2011).

Many ISGs function as direct antiviral effectors that prevent the viral genome replication, virion assembly or release from the infected cells. Others encode signalling pathways components such as PRRs or transcription factors in order to enhance the IFN response, thus creating a positive feedback loop, amplifying the antiviral response (Dixit and Kagan, 2013).

2.2 – Viral interaction with RIG-I/MDA-5 and subsequent recruitment of MAVS adaptor

2.2.1 – Activation of RIG-I by viral genomes and proximal signal transduction

The RLR activation is a multistep process that requires a well-coordinated interaction between receptor, ligand and several accessory proteins. Contrarily to RIG-I, the requirements for an efficient MDA5 activation are yet to be clarified, but it seems reasonable to assume that both follow a similar mechanism.

Taking RIG-I as an example, the knowledge of this process, to date, involves the following chain of events (as proposed by (Dixit and Kagan, 2013) review): **1 –** in resting-state cells, RIG-I adopts a closed conformation, resulting in an auto-inhibitory state (no signaling); **2** – pppRNA binds to RIG-I and induces conformational changes that lead to dimerization and exposure of CARDs in an open conformation; **3 –** phosphorylation events of RIG-I and TRIM25-dependent ubiquitination activate the signalling ability of RIG-I; **4 –** RIG-I associates with MAVS, in a CARD-dependent manner; **5 –** MAVS accumulates in signalling aggregates by a prion-like mechanism.

In the absence of infection, RIG-I is kept in an auto-inhibited state by intramolecular interactions between CARDs and helicase domains that hinders the RNA binding to the helicase domain and prevents CARDs from signalling (Kowalinski et al., 2011; Saito et al., 2007). When overexpressed, the N-terminal of RIG-I comprising the two CARDs adopts a constitutively active conformation, but, in physiologic conditions, the closed conformation only opens upon binding of the ligand at the C-terminus to facilitate the downstream signalling through CARDs (Kowalinski et al., 2011; Yoneyama et al., 2004).

Crystallographic structures of RIG-I present a detailed vision of the conformational changes triggered by the ligand binding, which are required to the signal initiation (Kowalinski et al., 2011; Luo et al., 2011). Structural data suggest a model where, in an auto-repressed state, the CTD is deprived of intramolecular interactions and can freely engage the binding of pppRNA (Kowalinski et al., 2011). This initial event increases the local RNA concentration and leads to a cooperative RNA and ATP binding to the helicase domain, resulting in dramatic rearrangements within the helicase domain (Kowalinski et al., 2011). The helicase domain and the CTD completely surround the RNA, anchoring themselves to the helix through a number of intramolecular interactions (Luo et al., 2011). This newly formed channel comprises 9 to 10 bp along

the RNA; longer RNAs allow the binding of two monomers of RIG-I, simultaneously (Kowalinski et al., 2011). However, this apparent dimerization lacks a protein-protein interface, reflecting instead an oligomerization guided by RNA (Kowalinski et al., 2011).

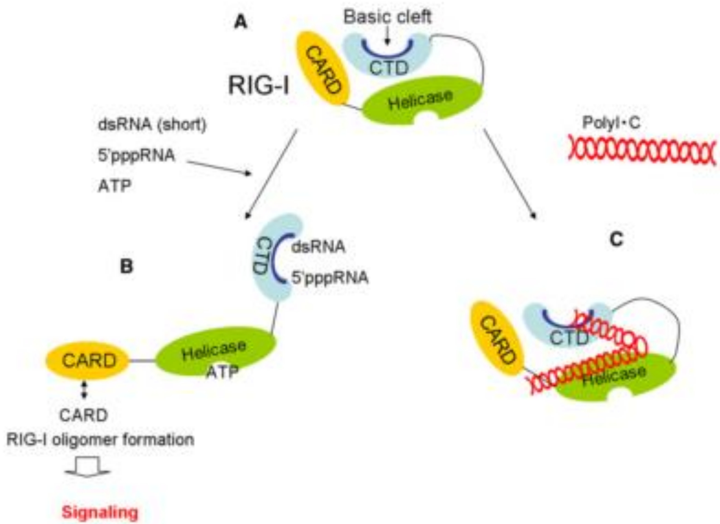

Figure 14 – Model of RIG-I activation by short dsRNA and/or 5'ppp ssRNA.
A) RIG-I, in its inactive state is found with a closed structure with the CARD repressed by, presumably, interactions between the CTD and helicase linker region. B) Upon viral infection, short dsRNA or 5'pppRNA activates RIG-I in the presence of ATP. C) Longer dsRNAs (like poly I:C) might interact with CTD and the helicase domain of RIG-I, allowing the formation of a stable complex that induces a distinct conformation of RIG-I (Takahasi et al., 2008).

The propagation of the signalling pathway through ligand-activated RIG-I is accomplished by the N-terminal CARDs (Yoneyama et al., 2004). Deletion of CARDs results in a dominant negative phenotype (Yoneyama et al., 2004). Take the following example: Huh7.5 cells, a subpopulation of Huh7 hepatocyte cell line, which is characterized by a mutation of threonine by an isoleucine in the position 55 (T55I) at the first CARD of RIG-I, failed to respond to HCV infection (Sumpter et al., 2005). As a consequence, the absence of a functional antiviral activity generated conditions for HCV replication in Huh7.5 cells (Sumpter et al., 2005). The T55I mutant interferes with the ubiquitin-ligase E3 TRIM25, which is necessary for the activation of RIG-I signalling (Sumpter et al., 2005).

Gack et al. reported that TRIM25 binds to the first CARD through its SPRY domain (Gack et al., 2007). A pre-requisite for TRIM25 is the phosphorylation of RIG-I at T170 by a (not yet identified) phosphatase (Gack et al., 2010). A phosphomimetic mutation of T170 jeopardized the TRIM25 binding to RIG-I and, subsequently, interfered with downstream signalling events (Gack et al., 2010). TRIM25 transfers ubiquitin fragments bound to K63 in lysine 172 (K172) at the second CARD, using its RING domain and

oligomerization of RIG-I with the MAVS adaptor depends critically upon this modification (Gack et al., 2007). Accordingly, TRIM25-defficient MEFs (Mouse Embryonic Fibroblasts) can't secrete IFN-β after infection with Sendai virus (Gack et al., 2007; Jiang et al., 2012). Despite the fact that TRIM25 does not bind ubiquitin fragments to MDA5, a poly-ubiquitin binding by MDA5 is necessary for its signalling functions (Jiang et al., 2012).

The need of RIG-I ubiquitination for the initiation of the downstream signalling was challenged by a study using a cell-free system, to identify the minimal components necessary for the signal transduction (Zeng et al., 2010). The RIG-I pathway was reconstituted by a mix containing affinity-purified RIG-I, raw mitochondria and peroxisomes (containing the adaptor MAVS), cytosolic extracts (containing TBX1), *in vitro* synthesised IRF3 and ATP. The RIG-I activity was quantified by the measurement of the IRF3 dimerization, a readout for its activation. In this *in vitro* assay, the authors reviewed key aspects of the RIG-I signalling and revealed new regulatory mechanisms. The IRF3 activation required MAVS and TRIM25, while depletion of these proteins by RNAi interfered with IRF3 dimerization. RIG-I needed to be isolated from virus-infected cells in order to be activated by *in vitro* RNA or to be present under the form of a N-terminal CARD fragment for the IRF3 activation to occur. The ubiquitination factory, responsible for RIG-I activation, showed to comprise E1, E2 Ubc5, Ubc13 and E3 TRIM25, once the mitochondrial fraction of infected cells with depletion of Ubc5 (isoform b and c) and Ubc13 no longer raised IRF3 dimerization. Thus, the TRIM25 requirement and K63-bound ubiquitin for the IFN-β induction by RIG-I was confirmed in this experimental context (Zeng et al., 2010).

As a consequence of the initial antiviral events (viral-RNA binding → RIG-I activation → CARDs ubiquitination) as previously described, RIG-I turn into an adequate complex, capable of inducing a powerful downstream signalling cascade through its interaction with the mitochondrial antiviral signalling (MAVS) adaptor.

2.2.2 – MAVS Interactome/Signalosome

The MAVS adaptor, comprising 540 amino acids, in *Homo sapiens,* is an external membrane protein with a predicted molecular weight of ~56kDa (Kawai et al., 2005; Meylan et al., 2005; Seth et al., 2005; Xu et al., 2005). MAVS is coded at the nuclear genome and it is ubiquitously expressed in a variety of cells lines and tissues (Kawai et al., 2005; Meylan et al., 2005; Seth et al., 2005; Xu et al., 2005).

Structurally, MAVS has a CARD domain in its amino terminal (aa. 10 to 77), a proline-rich region (PRR; aa. 107 to 173) and a C-terminal transmembrane domain (CTD; aa. 514 to 535) that anchors MAVS to the outer mitochondrial membrane (Seth et al., 2005). MAVS also comprises two TRAF-interacting motifs (TIMs) at the proline-rich region: one of them localizes at aa. 143-147 and binds TRAF2, and the second one localizes at aa. 153-158 binding TRAF6 (Seth et al., 2005; Xu et al., 2005). An alternative TRAF6-binding site is also located at the C-terminal (aa. 455-460), and both TIMs at the PRR and carboxyl terminal are required for the NF-kB activation mediated by TRAF6 (Xu et al., 2005). It was recently demonstrated that the C-terminal TIM of MAVS also binds to TRAF3 and exclusively mediates the induction of IFN and ISG expression (Paz et al., 2011; Saha et al., 2006).

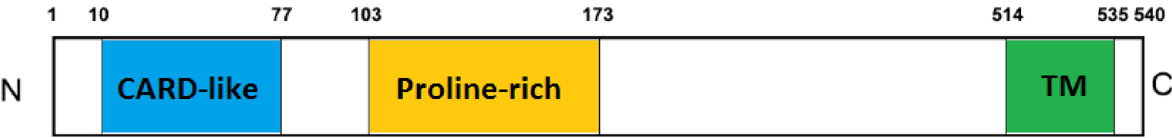

Figure 15 – Schematic representation of Mitochondrial Antiviral Signalling protein.
In accordance with (Seth et al., 2005); TM – Transmembrane domain; N- amino terminal; C-Carboxil terminal

The initial identification of MAVS was achieved by four independent groups, almost simultaneously (Kawai et al., 2005; Meylan et al., 2005; Seth et al., 2005; Xu et al., 2005). One group used a cloning strategy to identify activating molecules of IFN-β promoter and ended up isolating a gene, whose protein they named IFN-β Promoter Stimulator-1 (IPS-1) (Kawai et al., 2005). A second group isolated the same protein by screening of non-characterized proteins known as powerful NF-kB inducers; they named their protein as VISA – Virus-Induced Signalling Adaptor (Xu et al., 2005). Two other groups conducted research profiles at human proteins databases to identity novel proteins containing CARD domains, similar to those found at RIG-I and MDA5; they called the resulting products Cardif (CARD adaptor inducing IFN-β) and MAVS (mitochondrial antiviral signalling adaptor) (Meylan et al., 2005; Seth et al., 2005).

The precise space-time events that involve MAVS adaptor in viral infections are still under intense investigation. Although several studies portray adaptor molecules that interact with MAVS, it is unlikely that all of them have a physiological role at the MAVS mediated signalling [*MAVS interacting partners can be reviewed in* (West et al., 2011)]. However, given their physiological importance in the innate immune response to viral infection, some of the components of MAVS *interactome* already mentioned in this

chapter will be further analysed here, and a few more components will now be introduced.

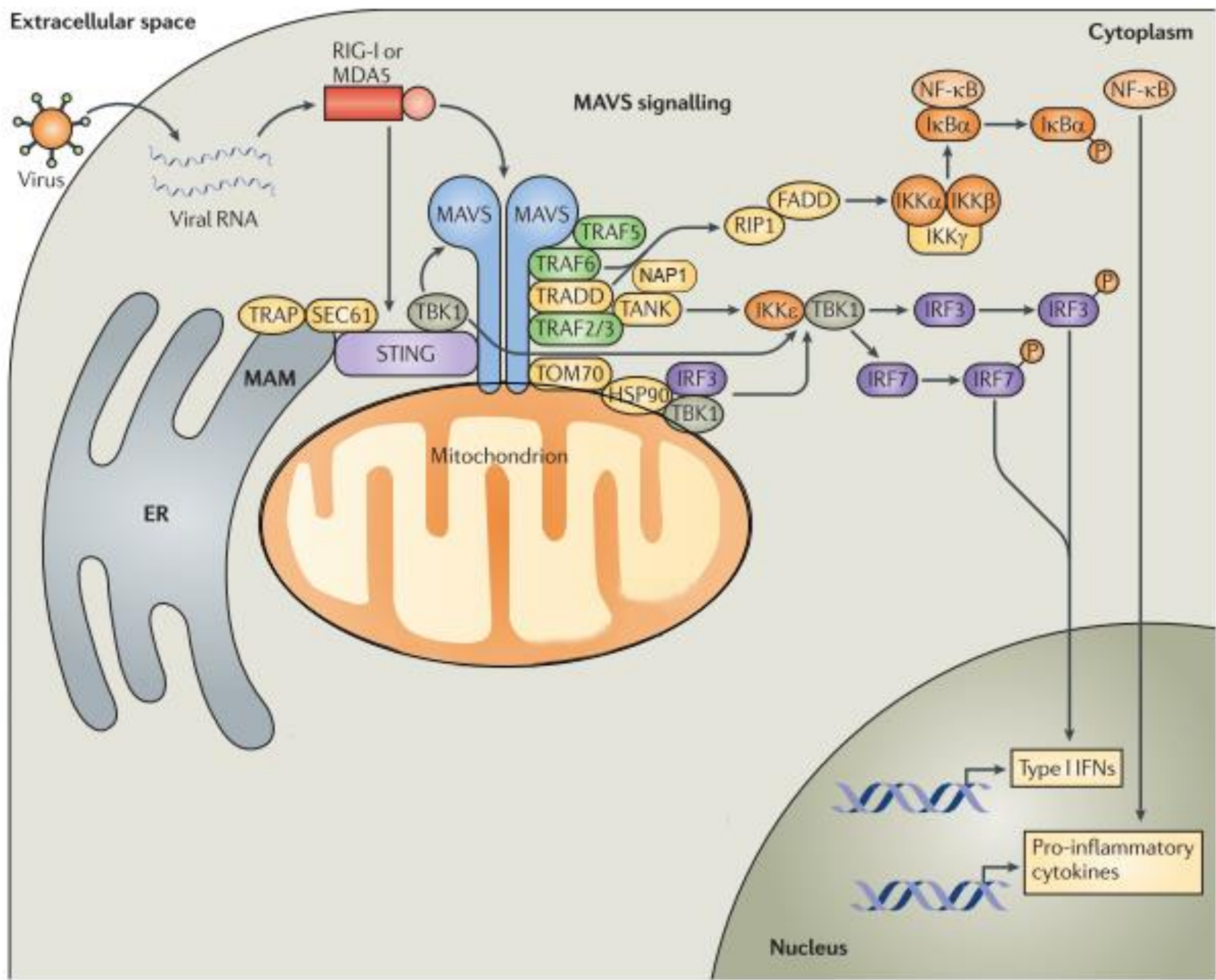

Figure 16 – Focusing on the Mitochondria: antiviral signalling pathways.
Cytosolic viral RNA is recognized by the RIG-I-like receptors (RLRs) RIG-I and MDA5, which activate MAVS through CARD-CARD interactions. MAVS then recruits various signalling molecules to transduce downstream signalling such as TRAF6 and TRAF5. TRAF6 along with TRADD activates canonical NF-kB signalling via RIP1 and FADD. Canonical NF-kB signalling occurs as the IκB kinase (IKK) complex – consisting of IKKα, IKKβ and IKKγ (a.k.a. NEMO) – phosphorylates the NF-kB inhibitor IκBα, resulting in the proteasomal degradation of IκBα and thus liberating NF-kB to translocate into the nucleus and initiate pro-inflammatory cytokine gene expression. MAVS also interacts with various molecules that activate IRF signalling (such as STING). These molecules, together with the TRAP complex and the SEC61 translocon mediate the activation of TBK1, which phosphorylates IRF3 and IRF7. In addition, MAVS interacts with TOM70 which, in turn, interacts with HSP90 and thereby localizes TBK1 and IRF3 in proximity to the MAVS signalosome. Finally, MAVS binds to TRAF2 and TRAF3 and through TRADD and TANK/NAP1 promotes IKKε- and TBK1-mediated phosphorylation of IRF3. This promotes IRF3 nuclear translocation, leading to the expression of type I interferon genes. Adapted from: (West et al., 2011)

TRAF3: The MAVS regulation of type I IFN induction is accomplished by direct and specific interaction between the TRAF domain of TRAF3 and a TRAF interaction motif of MAVS itself (TIM) (Saha et al., 2006; Tang and Wang, 2009). Point mutations in two critical amino acids (Y440A/Q442A) in the TRAF domain of TRAF3 prevented the association with MAVS and completely ablated the TRAF3-dependent IFN production, after viral infection (Saha et al., 2006). Additionally, TRAF3 knockout cells showed a pronounced reduction in IFN-α production, indicating its crucial role in RLR signalling (Saha et al., 2006). After TRAF3 recruitment to the signalling complex, the RING domain of TRAF3 – responsible for its E3 ubiquitin ligase activity – assembles Lys63 polyubiquitin chains, thus creating a scaffold for the assembly of a signalling complex made of IKKε and TBK1, leading to the activation of IRF3 and IRF7 ([reviewed at (West et al., 2011)].

TRAF2 and TRAF6: Similarly to TRAF3, TRAF2 and TRAF6 interact with MAVS (Xu et al., 2005). Although the binding of TRAF6 to MAVS has been demonstrated, the role of TRAF6 in IFN-α/β production is not fully understood. A recent report showed that NF-kB, JNK (c-Jun N-terminal kinase) and p38 mitogen-activated protein kinase activation was compromised in TRAF6-deficient MEFs in response to vesicular stomatitis virus (VSV) and poly I:C (Yoshida et al., 2008). Furthermore, this study suggested that MAVS requires TRAF6 and MEKK1 (mitogen-activated protein (MAP) kinase/extracellular signal-regulated protein kinase (ERK) kinase 1) to activate NF-kB and mitogen-activated protein kinases (MAPKs) to achieve an optimal type I IFN induction (Yoshida et al., 2008). In line with the role of TRAF6 in the RIG-I signalling, myeloid dendritic cells and fibroblasts, upon Sendai virus (SeV) infection, mediate the activation of the p38 MAPK, via TRAF2 and TAK1 (aka. Mitogen-activated protein kinase kinase kinase 7); the p38 MAPK activation, in turn, promoted the IL-12 production and increases the type I IFN induction (MIKKεlsen et al., 2009).

TANK and NAP-1: Both, TANK (TRAF-family member associated NF-kB activator) and NAP-1 (NF-kB-activating kinase-associated protein 1) are adaptor proteins involved in IRF activation in response to a viral infection (Guo and Cheng, 2007). TANK acts as a scaffolding protein with multi-domains that gathers elements of the IRF and NF-kB pathways, promoting the activation of both, the IKK complex and the IKK related kinases (Guo and Cheng, 2007). NAP-1 is an ubiquitous cytoplasmic protein that interacts with IKKε and TBK1, facilitating the IRF3 activation and induction of IFN-β after VSV infection (Sasai et al., 2006).

NEMO: Known as a regulatory subunit of the classical IKK complex, NEMO serves as a bridge for the NF-kB and IRF signalling pathways, promoting cross-talk between these pathways during RIG-I signalling to activate IFNs (Zhao et al., 2007). NEMO acts downstream of MAVS and interacts with TANK to mediate the recruitment of TBK1 and IKKε to the RIG-I – MAVS complex (Zhao et al., 2007). NEMO mutants, lacking the TANK-binding domain, fail to interact with IKKε, TBK1 and MAVS mediated signals (Zhao et al., 2007).

FADD, RIP1, TRADD and Caspases 8 and 10: Interestingly, FADD (Fas-associated death domain) and RIP1 (receptor interacting protein 1) have also been implicated in the antiviral pathway (Balachandran et al., 2004). These death domain containing molecules interact with MAVS C-terminal, inducing NF-kB through interaction and activation of caspases 8 and 10 (Takahashi et al., 2006). Both caspases are cleaved during dsRNA stimulation and overexpression of the cleaved form of these caspases is enough to activate NF-kB (Takahashi et al., 2006). In addition, caspase 8-deficient mouse derived cells showed reduced expression of inflammatory cytokines and NF-kB, illustrating the importance of these caspases in the NF-kB branch of the RIG-I signalling (Takahashi et al., 2006). Moreover, TRADD (Tumour necrosis factor receptor type 1-associated death domain), a fundamental adaptor of the TNFR-I (tumour necrosis factor receptor-1), has been showed to be recruited to MAVS, upon viral infection, coordinating the assembly of a signalling complex comprising TRAF3, TANK, FADD and RIP1, leading to IRF3 and NF-kB activation (Michallet et al., 2008)– see *Figure 16* above.

STING: The recent discovery of a novel protein, named stimulator of interferon genes (STING) brought a new component to the antiviral pathway (Ishikawa and Barber, 2008; Jin et al., 2008; Zhong et al., 2008). STING, comprising four transmembrane domains, was shown to be expressed at the external mitochondrial membrane and the binding of nucleic acids to RIG-I triggered the formation of a complex between MAVS and STING, conducting to the TBK1 recruitment and subsequent phosphorylation of IRF3 (Zhong et al., 2008). Conversely, the *Barber group* reported STING to be localized at the endoplasmic reticulum (ER) membrane through its transmembrane domains (Ishikawa and Barber, 2008). Although STING interacts directly with RIG-I, it was not able to co-immunoprecipitate with MDA5 or to respond to the poly I:C mediated IFN activation (Ishikawa and Barber, 2008). Other reported differences show that STING co-immunoprecipitates with TRAP complex components (TRAPβ - Target of RNAIII-activating protein beta) and the translocon adaptor SEC61β, whose cellular functions involve translocation of proteins through the

ER membrane, after translation (Ishikawa and Barber, 2008). This observation lead the authors to suggest that STING can bind RIG-I to the translocon, in order to detect translating viral RNAs to induce antiviral immunity through TBK1 (Ishikawa and Barber, 2008). *Jin et al.* localized a similar molecule, named MPYS, in both, mitochondria and the plasma membrane, and found an alternative function, where MPYS associates with the major histocompatibility complex class II (MHC II) and mediates apoptotic signal transduction (Jin et al., 2008). A full understanding of the role of STING/MPYS/MITA in innate immune signalling awaits further investigation.

2.2.3 – Posttranslational Modifications of MAVS

As mentioned in the beginning of this chapter, MAVS accumulates in the cell, in signalling aggregates, by a prion-like mechanism (Hou et al., 2011). Recent studies defined these aggregates as highly organised fibrils, self-replicating, like prions. Using a cell-free system to reconstitute RLR signalling in vitro, MAVS complexes, bigger than 26S proteasome were detected 9h after SeV infection, which coincided with IRF3 dimerization (Hou et al., 2011). These complexes present several prion features: (1) MAVS-CARD is required and it is enough for the formation of the fibril-like structures, as it was determined by electronic microscopy; (2) these fibrils are resistant to protease treatment and detergent solubilisation; (3) the protease resistant fibrils convert MAVS of mitochondria extracted from non-infected cells, into functional aggregates, leading to IRF3 activation (Hou et al., 2011). On the other hand, mitochondria lacking MAVS, generated by RNAi previous to the extraction, didn’t result in IRF3 dimerization (Hou et al., 2011). Importantly, MAVS aggregates form within minutes after RLR signal activation in the cell-free reconstitution, indicating that these MAVS fibrils are a bona fide determinant of the activated state of MAVS.

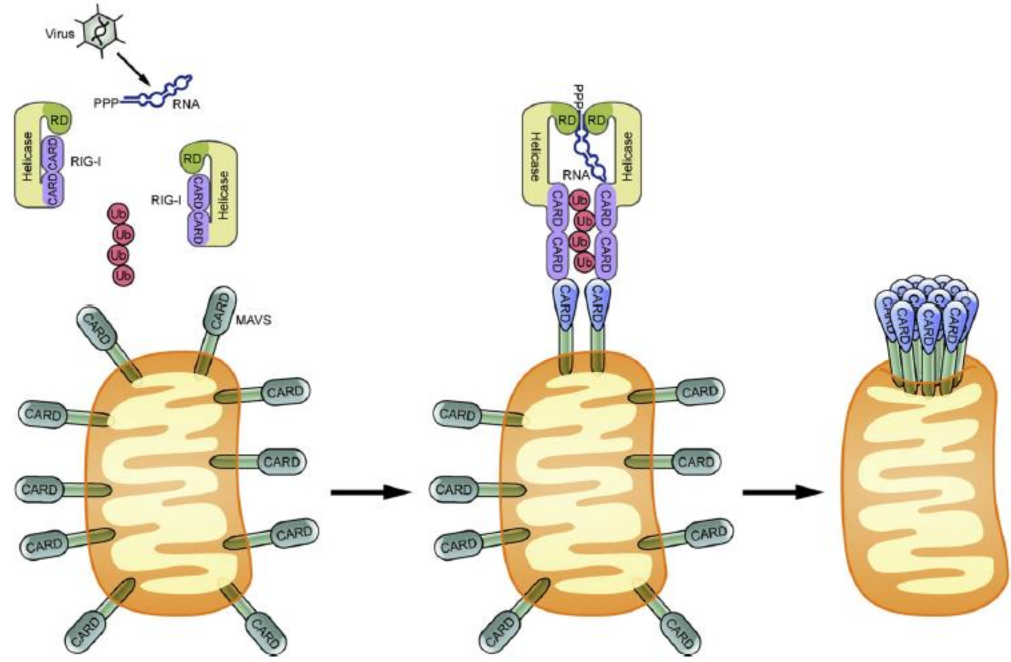

Figure 17 – MAVS forms prion-like aggregates when induced by RIG-I.
The interaction between CARD domains of RIG-I and MAVS induces a conformational change of the MAVS CARD, which in turn converts other MAVS on the mitochondrial outer membrane into prion-like aggregates. These aggregates activate cytosolic signalling cascades that turn on NF-kB and IRF3, leading to the induction of type I IFNs and other antiviral molecules (Hou et al., 2011).

Ubiquitination represents an important posttranslational modification of the proteins of the host cell, and the list of ubiquitinated proteins in RIG-I pathway is already extensive, demonstrating the relevance of this modification in the regulation of IFNs (Ribet and Cossart, 2010).

MAVS is ubiquitinated upon viral infection and studies show that K48 and K63 polyubiquitination occurs in virus-infected cells (Paz et al., 2009). Furthermore, polyubiquitination at lysine500 of MAVs leads to the recruitment of IKKε to MAVS and contributes to the negative regulation of IFN signalling by dislodging TRAF3 (Paz et al., 2009).

Several E3 ligases and proteins have been identified to contribute to the turnover of MAVS after viral infection: *You et al.* demonstrated that PCBP2 (Poly(rC)-binding protein 2) recruits the E3 ligase AIP4 (atrophin-1-interacting protein 4) to catalyse the K48 polyubiquitination and degradation of MAVS; *Zhong et al.* identified the E3 ligase RNF5 (ring finger protein 5) as another ligase that targets MAVS to ubiquitination and degradation (F. You et al., 2009; Zhong et al., 2010). In the latter study, Lys362 and Lys461 were identified as the K48 acceptor sites, which lead to proteasomal degradation of MAVS, upon viral infection (Zhong et al., 2010). *Arimoto et al.* identified

RNF125 (ring finger protein 125) as negative regulator of RIG-I pathway through a mechanism mediated by E2 ligase UbcH8 (a.k.a. UBE2L6 - Ubiquitin/ISG15-conjugating enzyme E2 L6) (Arimoto et al., 2007). Taken together, these studies provide evidence that the MAVS degradation is an important mechanism, employed by the cell to turn off the antiviral IFN response.

The negative regulation of MAVS signalling was also associated with phosphorylation. PLK1 (Polo-like kinase 1) was also identified as a MAVS partner, using yeast two-hybrid assays (Vitour et al., 2009). PLK1 associates with two discreet domains of MAVS, in a phosphorylation-dependent or independent manner (Vitour et al., 2009). The phosphodependent binding to MAVS requires phosphorylation at threonine 234 (Vitour et al., 2009). However, PLK1, as a negative regulator of MAVS, is independent of phosphorylation events and requires the binding of PLK1 to the MAVS C-terminal; this association disrupts the interaction of MAVS with TRAF3 (Vitour et al., 2009).

It has been showed that MAVS can be phosphorylated in the presence of IKKε, but not in the presence of TBK1, and the disruption of the MAVS – IKKε interaction, by point mutation at Lys500 of MAVS, diminishes, but doesn't completely abolish phosphorylation, suggesting other kinases to be involved in MAVS phosphorylation (Paz et al., 2009). A study by *Johnsen et al.* demonstrated that tyrosine-kinase c-Src augments the RIG-I mediated signalling at the level of TRAF3, although c-Scr can also interact with other components such as RIG-I and MAVS and, thus, participates in the formation of the innate signalling complex (Johnsen et al., 2009). c-Scr acts with MAVS and TRAF3 in the activation of IRF3, but the possibility of c-Scr directly phosphorylate MAVS, TRAF3 or RIG-I is yet to be determined (Johnsen et al., 2009). The non-receptor kinase protein like c-Scr, c-Abl has also showed to interact with, and phosphorylate MAVS, in order to regulate the immune response upon viral infection (Song et al., 2010). The same way as other adaptor proteins, the transmembrane and the CARD domain of MAVS are necessary for the interaction with c-Abl (Song et al., 2010). Further studies are necessary to delineate the specific sites of interaction/phosphorylation, as well as the physiological role of phosphorylation in this context (Song et al., 2010).

Like most of posttranslational modifications, phosphorylation is a reversible process, which involves the activity of phosphatase proteins. In this regard, the threonine-phosphatase EY4A was showed to interact with NLRX1 and STING, thus demonstrating their role in MAVS *interactome* (Okabe et al., 2009). Although several

studies have been demonstrated that MAVS is phosphorylated, there is no clear evidence of the relevance of this posttranslational modification for the IFN signalling or to the general outcome of viral infection.

2.3 - Host regulatory mechanisms of the virus – RIG-I/MDA5 – MAVS interface

The RLR signalling pathway is under tight control by a number of mechanisms to prevent an aberrant interferon production. The limitation of the IFN production is a physiological requirement for the general well-being of the organism. Upon initiation of antiviral responses, a restriction of the excess IFN production must occur. Therefore, several regulatory mechanisms take place to control the IFN levels, confirming the importance of counteracting the deleterious effects of IFNs, which include chronic cellular toxicity and the development of inflammatory or auto-immune diseases (Komuro et al., 2008; Loo and Gale, 2011; Ramos and Gale, 2011).

The first described regulatory mechanism was possible by the identification of a repressor domain within RIG-I and LGP2, which auto-regulates the RLR functions via dynamic intramolecular interactions(Komuro and Horvath, 2006; Rothenfusser et al., 2005; Saito et al., 2007; Yoneyama et al., 2005). LGP2 itself was found to play a role as positive and negative regulator of the RLR signalling pathway. Recent studies showed that, whereas LGP2-/- MEFs are more susceptible to IFN production stimulation in response to synthetic RNA (poly I:C), and LGP2-/- mice are more resistant to the lethal virus VSV, LGP2-/- cells/mice have a defective IFN production in response to EMCV (Satoh et al., 2010; Venkataraman et al., 2007). These apparently incongruent observations suggest that RIG-I may in fact be a target for the regulation of LGP2-mediated IFN production. The observed differences in LGP2 regulatory functions may be due, in part, to distinctions of genetic background of the mice lines, as well as the nature of each construct used to make the null LGP2 lines (Satoh et al., 2010; Venkataraman et al., 2007).

LGP2 has also been reported as a negative regulator of MAVS, through its direct interaction with MAVS at the mitochondrial membrane (Komuro and Horvath, 2006). This prevents the vital association of MAVS with IKKi (a.k.a. IKKε) for downstream signal propagation through IRF3 (Komuro and Horvath, 2006).

The regulatory functions of LGP2 are still to this date unclear and controversial between research groups and further work is required to determinate in which step of the RLR pathway LGP2 exerts its regulatory effects.

The regulatory mechanisms that will be further discussed in this chapter can be divided in three main categories: (1) **Regulation by protein-protein interactions** (where the previously described LGP2 regulation is included), (2) **regulation by posttranslational modifications of components and cofactors of the RLR signalling pathway** and (3) **regulation via organelle dynamics**.

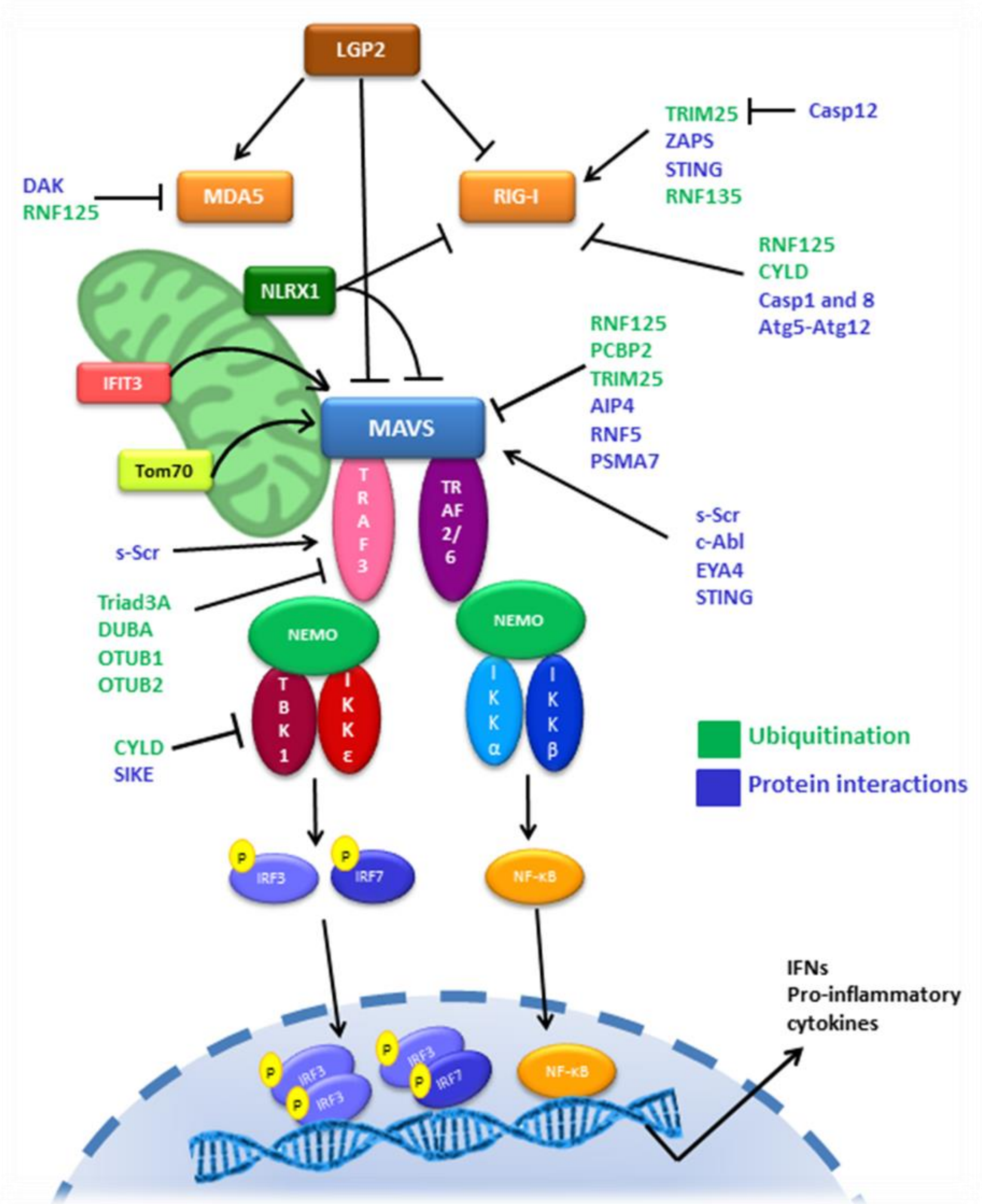

Figure 18 – RLR signalling cascade and its regulating partners.
Several cellular factors known to contribute to regulation of multiple stages of RLR signalling are depicted in the figure. LGP2 is the third member of the RLR family of proteins with contradicting roles in the RLR signalling pathway. IFIT3, NLRX1 and Tom70 are mitochondrial-localized

proteins which interact directly with MAVS. Factors involved in ubiquitin mediated regulation are denoted in green text; factors which require direct interaction with RLR signalling components are depicted in blue text.

2.3.1 – Regulatory protein interactions

Regulation of the RLR signalling components via protein interactions serves as one of the control mechanisms of the RLR response to a given viral infection. This regulation is mainly achieved through negative feedback, in order to regulate the levels of IFN production in later states of the cellular immune response, but there are also a few positive feedback mechanisms.

In fact, **ZAPS** protein (zinc finger antiviral protein shorter isoform) directly associates with RIG-I in a ligand-dependent manner to potentiate and amplify downstream signalling events, such as IRF3 and NF-kB activation and type I IFN induction (Hayakawa et al., 2011). Additionally, tyrosine kinase **s-Scr** interacts with MAVS, TBK1 and TRAF3, likely within the MAVS signalosome to enhance RLR-dependent IFN induction (Johnsen et al., 2009). Separately, **c-Abl** (Src-like non-receptor protein kinase) has been showed to interact with, and phosphorylate MAVS during viral infection, to facilitate both signalling of IRF3 and NF-kB (Song et al., 2010). Besides, **EYA4** (Eyes Absent 4) and its phosphothreonine-specific phosphatase activity are required to facilitate IFN signalling. EYA4 has been reported to interact with MAVS, STING and NLRX1, more likely within MAVS signalosome (Okabe et al., 2009).

The mitochondrial protein **Tom70** (Translocase of outer membrane 70) has been identified, through mass spectrometry and immunoprecipitation, as a novel MAVS-interacting partner (Liu et al., 2010). Tom70 is a member of the TOM complex at the outer mitochondrial membrane that recognizes newly synthesised mitochondrial proteins at the cytosol and has a role in the translocation of such proteins to their destination within the mitochondria [reviewed at (Baker et al., 2007)]. Exogenous Tom70 potentiated IFN-β induction in response to SeV and cytosolic poly I:C stimulation, while its depletion by RNAi resulted in an abrogated IFN-β induction to the same stimuli (Liu et al., 2010). MAVS and Tom70 interaction was enhanced in response to SeV infection and poly I:C and it requires the mitochondrial localization of both proteins (Liu et al., 2010). Based in previous works by the same group showing that chaperone protein **Hsp90** was constitutively complexed with IRF3/TBK1 (Yang et al., 2006), and other studies showing that Hsp90 interacts with Tom70 (Young et al., 2003), the authors proposed the hypothesis that Tom70 can be involved in the

recruitment of IRF3/TBK1 to MAVS to enable signalling (Liu et al., 2010). In fact, immunoprecipitation experiments confirmed that exogenous Hsp90 bound to Tom70 and IRF3/TBK1 and exogenous Tom70 interacts with Hsp90 and IRF3/TBK1 (Liu et al., 2010). The results of these studies taken together proposed a model in which Tom70 interacts with MAVS and recruits both, IRF3/TBK1 and IKK complexes to mitochondria, by specifically recognizing and interacting with Hsp90, thus linking MAVS to downstream signalling.(Liu et al., 2010)

Other studies have demonstrated that **ISGs** can also regulate MAVS to influence signalling. The **IFIT3** protein (IFN-induced protein with tetratricopeptide repeats 3) is an ISG and represents a candidate to positive feedback in RLR signalling, via MAVS interactions (Liu et al., 2011). The IFIT proteins are known for being highly inducible by viral infections and IFNs. IFIT3 localizes at mitochondria, where it interacts with both MAVS and TBK1. Overexpression of IFIT3 potentiated RLR signalling, while its depletion reduced it. IFIT3 probably acts as a scaffold to favour MAVS interaction with TBK1, since this interaction is weak in the absence of IFIT3 and reinforced in its presence. Thus, this study added IFIT3 to the list of positive regulators of MAVS signalosome and found a novel role of IFIT3 in the antiviral signalling (Liu et al., 2011).

Conversely, a greater number of protein interactions acts as negative feedback regulatory factors. For instance, a proteasome molecule, the subunit **PSMA7(a4)** has been shown to directly bind to MAVS and reduce its ability to translocate downstream IFN signals. This regulation operates through a MAVS proteasomal degradation-dependent mechanism (Jia et al., 2009). The autophagy conjugate **Atg5-Atg12** has been shown to regulate RLR signalling via direct interactions with both CARD domains of RIG-I and MAVS to limit the downstream IFN production (Jounai et al., 2007). **SIKE** (Suppressor of IKK-epsilon) is a physiological suppressor of TBK1 and IKKε that keeps these kinase proteins sequestered in inactive complexes to prevent non intentioned activation by any RLR (Huang et al., 2005).

The specific regulation of MDA5 through protein interactions is less understood. However, MDA5 regulation has been described to occur via **DAK** (dihydroacetone kinase), in which, the overexpression of this molecule leads to diminished IFN responses to MDA5 agonists. Besides, DAK was found to interact with MDA5 but not with RIG-I, suggesting that the mechanism for the activation acts in order to prevent MDA5 activation and its interaction with MAVS. There's still a requirement for further comparison studies between the regulatory mechanisms of RIG-I and MDA5, with the

purpose of defining specific regulatory processes that take place via protein interactions (Diao et al., 2007).

Recent studies identified unique roles for some members of the caspase family in the regulation of RLR signalling (Kim and Yoo, 2008; Takahashi et al., 2006; Wang et al., 2010). This family of proteins perform in the activation of apoptotic cascades [reviewed at (Fan et al., 2005)] as well as targeting the inflammasome activation through processing and liberation of IL-1β, IL-18 and IL-33 [reviewed at (Kanneganti, 2010)].

Caspase 1, whose functions as inflammatory caspase and whose expression is activated during viral infections, was demonstrated to negatively regulate RIG-I signalling by promoting RIG-I secretion, therefore controlling its intracellular levels (Kim and Yoo, 2008). It is yet not clear how RIG-I is secreted but, since RIG-I interacts with caspase 1 and it was found in supernatant together with caspase 1, an hypothesis arises, stating that RIG-I secretion involves the inflammasome complex. However its physiological significance has not yet been clarified (Kim and Yoo, 2008).

Caspase 8 has shown to associate with MAVS signalosome via interactions with FADD, RIP1 and MAVS, after dsRNA stimulation (Rajput et al., 2011; Takahashi et al., 2006). Caspase 8 can function by supressing RLR signalling via two distinct mechanisms. First, caspase 8 promotes direct depletion of RIG-I through a mechanism dependent on its ability to bind to FADD, and independent of its cleaving ability. Second, caspase 8 cleaves RIP1 at signalosome complex, leading to the loss of IFN induction. These roles of caspase 8 are thought to act in the negative regulation and terminate RLR response in later stages after the initial RLR triggering during an acute viral infection. However, a further understanding of the RIP1 cleavage mechanism is needed for a better comprehension of how this process regulates MAVS signalosome and RLR functions (Rajput et al., 2011).

Caspase 12 has also been identified as a negative regulator of RLR signalling (Wang et al., 2010). Using a West Nile virus (WNV) infection model, caspase12-/- mice showed higher mortality rates to viral infection. Besides, knockout mice cells failed to mediate TRIM25 ubiquitination of RIG-I, and this correlates with the decrease in IFN responses and lack of protection against WNV. The exact mechanism by which caspase 12 regulate TRIM25 has yet to be described, requiring further analysis. Additionally, since only a small percentage of the human population expresses caspase 12, its role in RLR regulation remains debatable. However, this study

identified the potential cross-talk between the RLR and caspase signalling pathways as regulatory features that govern the antiviral immune response (Wang et al., 2010). Since the CARD-CARD interactions play an important role in both, RLR and caspase pathways, it is likely that more regulatory interactions will be identified in a near future.

Proteins localized at mitochondria are logical candidates for MAVS regulation. The first mitochondrial protein identified as a negative regulator of MAVS was **NLRX1** (nucleotide-binding oligomerization domain, leucine rich repeat containing X1) (Moore et al., 2008). NLRXs are members of the NOD-like cytosolic receptors family of pattern recognition receptors, which initially were thought to be independent of RLR signalling. This study confirmed the putative localization of NLRX1 at the outer mitochondrial membrane and went on by showing that NLRX1 interacts with MAVS through its CARD domain, disrupting upstream interactions with MAVS interacting partners (Moore et al., 2008). This data was corroborated in NLRX1-/- MEFs: IFN-β production was enhanced in NLRX1 knockout MEFs infected with a variety of viruses known to induce RIG-I (Allen et al., 2011). Interestingly, NLRX1-deficient cells exhibit RIG-I–MAVS association, even in the absence of viral infection. This constitutive association between RIG-I and MAVS in the absence of NLRX1 may explain the increase of IFN-β response to a viral infection that uses RIG-I but not with viruses that require MDA5 (e.g. encephalomyocarditis virus) (Allen et al., 2011). There are, however, contradicting results, since two studies using NLRX1 knockout MEFs, independently showed no potentiation of IFN induction or IRF3 phosphorylation in response to poly I:C or Sendai virus (SeV), in comparison with wild-type MEFs under poly I:C infection (Rebsamen et al., 2011; Soares et al., 2012). Another study reported that inhibition of the RLR signalling pathway may be an artefact of inhibition of the luciferase activity, which is quite relevant, since many of the previously mentioned studies used luciferase based assays to measure RLR signalling (Ling et al., 2012). Like the LGP2 inhibition of MAVS activity, NLRX1 have led to contradicting results. It is plausible that NLRX1 possesses several regulatory roles, depending on which ones are advantageous for the cell. Further studies are required to reconcile these findings and clarify the exact role of NLRX1 in MAVS signalling.

STING, also known as MITA, MPYS or ERIS, was originally identified as a RIG-I signalling regulator, owing its ability of direct binding to RIG-I, MAVS and TBK1 to its knockout phenotype (Ishikawa and Barber, 2008; Jin et al., 2011; Sun et al., 2009; Zhong et al., 2008). Overexpression of active fragments of RIG-I failed to induce IFN in STING-deficient MEFs. Besides, VSV infection in STING-deficient mice resulted in

significantly reduced survival rates and lower IFN serum levels, comparatively to control mice (Ishikawa and Barber, 2008). Thus, STING has been found to play an indisputable role in IFN response to cytosolic DNA from viruses or synthetic agonists.

Along with the mitochondria-resident proteins, those who relocate to mitochondria during a given viral infection also represent a cluster of MAVS regulators. Complement protein **gC1qR** (receptor for globular head domain of complement component C1q) was investigated for its role in antiviral signalling, due to reports that it is involved in cytomegalovirus and rubella infections (Xu et al., 2009). gC1qR localizes at mitochondria, nucleus, cytoplasm and cell surface. Interestingly, it has been demonstrated that gC1qR is recruited to mitochondria upon viral infection or poly I:C stimulation. Overexpression of gC1qR inhibits antiviral signalling activated by virus, poly I:C or exogenous RLRs. In addition, gC1qR and MAVS interact weakly in non-infected cells; this interaction was strongly enhanced upon viral infection. gC1qR knockdown, mediated by RNAi, resulted in an increased antiviral response to exogenous RIG-I or VSV infection (Xu et al., 2009). This study revealed a role for this complement receptor, which is known to act in several branches of innate immunity, in the regulation of RLR signalling through its interaction with MAVS.

2.3.2 – Regulation by posttranslational modification events

The posttranslational control of proteins is a common mean by which cells regulate a diversity of pathways and processes. Thus, it is not surprising that posttranslational modifications of RLR signalling pathway components are a key aspect in the cell antiviral signalling regulation. Specific modifications of RLRs and their co-factors impose regulation of signalling of the immune defences in a variety of levels, ranging from RLR activity to the assembly and function of MAVS signalosome.

Posttranslational modifications such as ubiquitination or deubiquitination are a major point of the RLR regulation. **RNF135** protein (RING finger protein leading to RIG-I activation or Riplet) is an ubiquitin-ligase that interacts with RIG-I but not with MDA5 (Oshiumi et al., 2009, 2010). It is reported to mediate the conjugation of K63-linked polyubiquitin chains to RIG-I at its C-terminal domain, as well as in its repressor domain, and it is crucial to the virus-induced IFN signalling (Oshiumi et al., 2009). RNF135-deficient mice weren’t able to produce IFNs or cytokines during a RNA virus infection, and subsequently, those mice were more susceptible to viral infection than

wild-type mice (Oshiumi et al., 2010). **TRIM25** also mediates K63-linked polyubiquitination of RIG-I at aa. K172 upon viral infection (Gack et al., 2007). This modification is thought to stabilize RIG-I and MAVS interactions to induce the activation of IFN production (Gack et al., 2007). However, **free K63-polyubiquitin chains** are also able to induce RIG-I activation in an *in vitro* reconstitution of the RIG-I pathway, suggesting that it is the actual polyubiquitin chain binding at K172 and not the ubiquitin-induced change that leads to RIG-I activation (Zeng et al., 2010). This data suggested that K63-polyubiquitin chains act as the second ligand to increase activation of RIG-I signalling (Zeng et al., 2010).

RIG-I signalling is also subject of regulation by the ubiquitin editing protein **A20** (Lin et al., 2006). A20 has both ubiquitin-ligase and deubiquitinase activities; however, only the ubiquitin-ligase activity, linked to its C-terminal, is important for the RLR signalling regulation. Overexpression of A20 inhibits RIG-I-dependent activation of IRF3 and NF-kB, while its depletion increased virus-induced signalling, suggesting its role as a negative regulator of RLR pathway (Lin et al., 2006). Additionally, TRAF3, a K63-linked ubiquitin-ligase and essential regulator of IRF3 activation induced by viral infection, was found to regulate IFN but not inflammatory cytokine production during viral infection (Häcker et al., 2006). TRAF3 binds to TIM (TRAF-interacting motif), which is found at the proline-rich region of MAVS and facilitates the IKKε recruitment to the MAVS signalosome (Saha et al., 2006). TRAF3 activity in RLR signalling is further regulated by the E3 ubiquitin-ligase **Triad3A** (Nakhaei et al., 2009), the **OTUB1**, **OTUB2** (Li et al., 2010) and **DUBA** (Kayagaki et al., 2007) deubiquitinases, and the interferon-stimulated gene **FLN29** (Sanada et al., 2008), alongside with the stability of the interactions between IKKε and MAVS (Paz et al., 2009) and by the PLK1 (Polo-like kinase 1) – MAVS interactions (Vitour et al., 2009).

RNF125 is another ubiquitin-ligase that cooperates with E2 ubiquitin-ligase HbcH5 to conjugate K48-linked ubiquitin to RIG-I, MDA5 and MAVS in order to mediate proteasomal degradation (Arimoto et al., 2007). RNF125 is an ISG itself, whose expression is induced after viral infection and its action is part of a negative feedback loop to prevent an excessive IFN production. RNF125 activity is supressed by **UbcH8** – the same ubiquitin-ligase responsible for the conjugation of ISG15 to target proteins during a viral infection (Arimoto et al., 2008). Based on cumulative evidence, it was proposed that the interaction of ISG15 with UbcH8, simultaneously with virus-induced ISG15 expression, dissociates from its interaction with RNF125. This action will then facilitate the ubiquitin conjugation by RNF125 at RIG-I and other molecules to inhibit

RLR signalling and IFN expression (Arimoto et al., 2008). Consistent with this model, basal expression of RIG-I is higher in Ube1-deficient cells that fail in their ability to conjugate ISG15, comparatively to wild-type cells, providing RIG-I amounts that facilitate a robust RLR signalling (Arimoto et al., 2008). In addition, the tumour suppressor **CYLD** is a deubiquitinase that interacts with both RIG-I and MAVS (Zhang et al., 2008). It was previously shown to be essential to prevent aberrant activation of IKKε and TBK1 (Zhang et al., 2008). Consistent with this finding, CYLD expression-deficient cells signal a constitutive activation and exhibit an hyper-induction of IFN during viral infection (Zhang et al., 2008). Besides, a recent study suggests that CYLD operates in the removal of polyubiquitin chains from RIG-I and TBK1 to inhibit IRF3 signalling and further IFN production within the MAVS signalosome (Friedman et al., 2008). Taken together, these studies define a critical role for reversible ubiquitination of RLRs and co-factors at the RLR signalling pathway during an immune response to a viral infection.

RLR signalling pathway is also regulated by additional posttranslational modifications, including phosphorylation, acetylation and SUMOylation. Aside from the previously described modification events that govern ubiquitination, further posttranslational modifications of RIG-I serve as means of prevention of its premature activation in the absence of PAMPs, and represent another level of regulation of the RLR activation. For instance, **casein kinase II** phosphorylates RIG-I at its resting state at threonine 770, serine 854 and 855 (Sun et al., 2011). Mutations at these sites, chemical inhibition or casein kinase II depletion turn RIG-I into a constitutive active state, resulting on an augmented IFN induction. By contrast, treatment of cells with phosphatase inhibitor supresses RLR-dependent signalling, suggesting that RIG-I phosphorylation is required to maintain the auto-regulation of its repressor domain (Sun et al., 2011). RIG-I was additionally identified to be acetylated in its K858 and K909 residues; however, it remains undetermined how acetylation of lysine regulates RIG-I signalling activity (Choudhary et al., 2009).

Downstream components of RLR signalling are also regulated by posttranslational modifications. Of note, the virus-induced activation of TLR and RLR pathways are known to lead to SUMOylation of IRF3 and IRF7 at their K152 and K406, respectively (Kubota et al., 2008). Mutants of these factors that cannot support SUMO modification exhibit an increased IFN induction, suggesting that the SUMO modification of IRF3 and IRF7 is a negative regulation mechanism (Kubota et al., 2008).

The specific posttranslational modifications of MAVS were already introduced in the previous chapter as well as the present one, but further discussion is required in order to approach some novel regulatory mechanisms of RLR signalling.

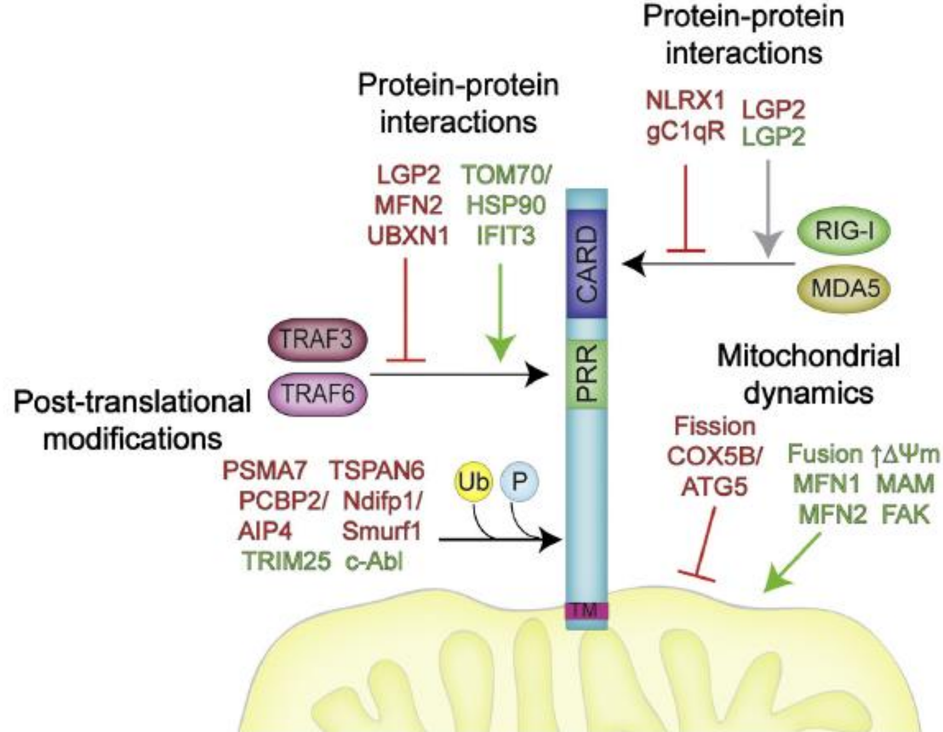

Figure 19 – Mechanisms of MAVS regulation.
MAVS can be regulated by host cell factors that inhibit MAVS signalling by direct protein-protein interactions, by altering mitochondrial properties or dynamics, or by post-translational modifications. PRR – proline-rich region; Ub – ubiquitination; P – phosphorylation. Positive regulators of MAVS signalling are shown in green text and negative regulators of MAVS signalling are shown in red text. Note that LGP2 is shown in both red and green given the confliting results on its role in the regulation of RLR signalling. (Jacobs and Coyne, 2013)

PCBP2 acts as negative regulator of MAVS, mediating its ubiquitination and proteasomal degradation (Fuping You et al., 2009). PCBP2 is involved in DNA and RNA binding with several purposes within the cell, including mRNA stability and regulation of protein translation (Makeyev and Liebhaber, 2002). Overexpression of PCBP2 results in the suppression of IFN-β induction mediated by MAVS but has no effect in TBK1 – IRF3 signalling. PCBP2 was highly induced under interferon treatment and viral infection, and the interaction between endogenous PCBP2 and endogenous MAVS was induced by SeV infection. Subcellular localization studies showed that PCBP2 localizes primarily at the nucleus but re-localizes to the cytoplasm, where it co-localizes with MAVS upon viral infection or MAVS overexpression. Despite not having any ubiquitin kinase activity by itself, PCBP2 overexpression induces MAVS proteasomal degradation. Through mutational analysis of MAVS, the authors showed

that ubiquitination of two specific residues of lysine lead to MAVS degradation and revealed that polyubiquitination levels of MAVS where higher in the presence of overexpressed PCBP2. Given that PCBP2 isn't an ubiquitination enzyme, the authors proposed that PCBP2 can act as a scaffold, binding MAVS to an E3 ubiquitin-ligase (Fuping You et al., 2009).

Tracing for known E3 ubiquitin-ligases for a potential candidate that mediates MAVS degradation and binds to PCBP2, the authors found the E3 ubiquitin-ligase **AIP4**, a Nedd4-like E3 ubiquitin-ligase (Fuping You et al., 2009). Overexpression of AIP4 partially interrupted IFN-β signalling and induced MAVS degradation, in an ubiquitin-ligase activity-dependent manner. Despite being demonstrated that AIP4 interacts with MAVS, this interaction requires PCBP2, which suggests that PCBP2 actually functions as a scaffold to facilitate the AIP4-dependent MAVS degradation. This was confirmed using *in vitro* ubiquitination assays, which showed that PCBP2 expression greatly increases AIP4-mediated ubiquitination and, consequently, MAVS degradation. At last, IFN-β signalling was increased in mice Itch-/- MEFs (Itch is the mice AIP4 homolog), further connecting this E3 ligase to MAVS signalling. Collectively, this study proved that PCBP2 acts as an adaptor for AIP4-mediated MAVS ubiquitination, and subsequent proteasomal degradation of MAVS in order to negatively regulate the RLR pathway, lighting up a novel mechanism for RLR pathway regulation (Fuping You et al., 2009). A later study by the same group has demonstrated that **PCBP1** is also involved in MAVS negative regulation, using a similar mechanism (Zhou et al., 2012). However, and unlike PCBP2, PCBP1 doesn't induce type I IFNs, leading the authors to conclude that PCBP1 acts as a "housekeeper" for MAVS levels and is not exactly a negative feedback inhibitor (Zhou et al., 2012).

Besides acting in RIG-I specific regulation, **TRIM25**-dependent ubiquitination also plays a role in regulation of MAVS through a negative feedback mechanism different than other ubiquitin-ligases (Castanier et al., 2012). TRIM25 overexpression enhanced MAVS ubiquitination and degradation, and catalysed the addition of ubiquitin in two specific residues of MAVS (K7 and K10). Functional studies revealed that exogenous TRIM25 increases IFN-β promoter activity, stimulated by high molecular weight poly I:C (a known MDA5 agonist used to avoid experimental complications related with TRIM25 modification of RIG-I) and, conversely, TRIM25 depletion lead to attenuation of IFN-β promoter activity, due to a diminished ubiquitination and degradation of MAVS. Based in these observations, the authors proposed that, under RLR activation, MAVS signalosome was assembled at the mitochondria, and only under TRIM25-mediated

ubiquitination and proteasomal degradation MAVS can be released and translocate to the cytosol to phosphorylate IRF3 and subsequently induce IFN-β production. While this presents as a tempting hypothesis, it remains unverified if the degradation elimination of MAVS (perhaps through mutational analysis of the MAVS ubiquitinated residues) maintains the same effect in the IRF3-mediated IFN-β induction (Castanier et al., 2012).

Ndfip1 has also been classified as a negative regulator of MAVS at mitochondria via an increase in ubiquitination and proteasomal degradation (Wang, Tong, and Ye, 2012). In light of cumulative evidence establishing connections between E3 ubiquitin-ligase activity with MAVS regulation, Ndfip1 is a logical candidate, given its role in the enhancement of protein ubiquitination through interactions with the E3 ubiquitin-ligase family known as Nedd3 ubiquitin-ligases. Ndfip1 inhibited MAVS-mediated signalling in a proteasome-dependent manner. Since these results pointed to proteasomal degradation mediated by ubiquitination as the regulatory mechanism of MAVS, the authors performed a screening of other four members of Nedd4 E3 ubiquitin-ligase family, for their ability to induce MAVS degradation, but not RIG-I or TBK1, in the presence of Ndfip1. Nedd4 E3 ubiquitin-ligase **Smurf1** led to MAVS degradation but not RIG-I/TBK1, in the presence of Ndfip1. Smurf1 and MAVS interaction was enhanced in the presence of Ndfip1, as well as the Smurf1-mediated MAVS ubiquitination, indicating that Ndfip1, probably, serves as an adaptor for the recruitment of Smurf1 to MAVS. This study described a regulatory mechanism similar to PCBP2 and AIP4 and provides another example of the complexity of ubiquitination in MAVS signalling regulation (Wang, Tong, and Ye, 2012).

TSPAN6 (tetraspanin protein 6) was recently described for its role in RLR-mediated MAVS signalling (Wang, Tong, Omoregie, et al., 2012). TSPAN6 is a member of the membrane-embedded tetraspanin protein family that have been showing several roles within the cell, including a variety of roles in host immunity [reviewed at (Hemler, 2005)]. Interestingly, TSPAN6 does not promote direct MAVS ubiquitination but it is ubiquitinated itself, in order to promote its association with MAVS and to disrupt MAVS signalosome at mitochondria. Overexpression of TSPAN6 resulted in a reduction of exogenous MAVS-induced signalling and showed interaction with MAVS. The authors proposed that TSPAN6 ubiquitination, in the presence of viral infection, promotes its recruitment to the mitochondria, where it interacts with MAVS, disrupting the assembly of MAVS signalosome, thus inhibiting antiviral signalling. The responsible enzymes for

TSPAN6 ubiquitination, in the context of RLR activation, are yet to be determined (Wang, Tong, Omoregie, et al., 2012).

Just like ubiquitination, **phosphorylation** represents a posttranslational mechanism of protein regulation of a variety of cellular processes such as the RLR signalling. A recent report revealed that **UBNX1** (ubiquitin regulatory X domain-containing protein) negatively regulates MAVS by binding and blocking its interaction with TRAF3/TRAF6 at their binding site at MAVS C-terminal, supporting even further the importance of this region in MAVS mediated signalling (Wang et al., 2013). More recently, tyrosine kinase **c-Abl** was identified as MAVS interacting partner, acting as its positive regulator by direct interaction and phosphorylation (Song et al., 2010). c-Abl is a nuclear and cytoplasmic Src-like non-receptor protein tyrosine kinase known to play different roles within the cell [reviewed at (Backert et al., 2008; Koleske, 2006)]. MAVS–c-Abl interaction has shown to require both transmembrane domain and CARD domain of MAVS, probably suggesting that MAVS mitochondrial localization is fundamental for this interaction. c-Abl depletion results in an abrogated MAVS signalling and pharmacological inhibition of c-Abl disrupts IFN-β production in response to VSV infection. Tyrosine phosphorylation of MAVS was increased by c-Abl expression but not by a c-Abl mutant lacking its kinase activity ability (Song et al., 2010). In a later report, tyrosine-scanning mutational analysis revealed that **inducible phosphorylation at MAVS Tyr9 residue** is involved in the recruitment of TRAF3/TRAF6 to propagate MAVS-mediated RLR signalling (Wen et al., 2012). If c-Abl is involved in Tyr9 phosphorylation of MAVS is yet to be determined and might represent an interesting follow-up for these two studies.

2.3.3 – Organelle dynamics as regulatory mechanisms of RLR signalling pathway

All three receptors of RLR family are cytosolic proteins and haven't been associated to any subcellular structure in steady-state. However, several signalling components downstream of these receptors are membrane proteins, whose functional domains project to the cytosol from the surface of the respective organelles. More important, the correct localization of such proteins is a requirement for their biological activity, and the physical proprieties of the organelles where they reside may play a role in the regulation of their activities. The best characterized example in this context is the adaptor protein MAVS.

Initial evidence for the role of mitochondrial dynamics in MAVS signalling came from studies demonstrating that SeV-infected cells or poly I:C transfection resulted in mitochondrial elongation/fusion, leading the authors to conclude that activation of the RLR signalling pathway results in physical alterations in mitochondria themselves (Castanier et al., 2010). In fact, the same study also demonstrated that phosphorylation of IRF3 was delayed in cells with fragmented mitochondria and that RLR signalling was attenuated by mitochondrial fragmentation and increased by mitochondrial fusion. Immunoprecipitation experiments revealed that MAVS interacts with **MFN1** (mitofusin 1), a protein responsible for the regulation of mitochondrial fusion events, suggesting a possible role of this interaction in the mitochondrial dynamics regulation that accompanies antiviral signalling (Castanier et al., 2010). Interestingly, a later report about MAVS – MFN1 interaction showed that MFN1 acts as a positive regulator of MAVS-mediated antiviral signalling by redistributing MAVS to speckle-like aggregates, observed after RLR signalling activation. This can explain why MFN1 and mitochondrial fusion seem to be important in RLR signalling, given that mitochondria may facilitate MAVS aggregation (Onoguchi et al., 2010). Others have investigated the role of MFNs in MAVS signalling using MFN1/MFN2-deficient MEFs; these cells were unable to undergo mitochondrial fusion and presented a compromised ability to produce IFN-β and IL-6, in response to a given viral infection (Koshiba et al., 2011). In line with these results, it is likely that the role of MFNs in the innate immune signalling is versatile. Not only MFN1 and MFN2 can directly interact with MAVS to exert a regulatory role, but their activities in mitochondrial dynamics appear to be important in MAVS functions. This is in accordance with previous studies, where MAVS activation required auto-association in higher order oligomers and the formation of large prion-like aggregates to induce powerful antiviral signal propagation (Hou et al., 2011; Tang and Wang, 2009). Recently, prion-like aggregates containing MAVS were reported to be responsible for downstream signal propagation by binding to TRAF2, TRAF5 and TRAF6 in an ubiquitin-dependent manner. This study also provides evidence that TRAF2 and TRAF5 act redundantly with TRAF6 to activate IRF3 (Liu et al., 2013).

Additionally, MFN1 and MFN2-deficient cells exhibit a dissipated mitochondrial membrane potential, suggesting that mitochondrial membrane potential may be important in RLR antiviral response (Koshiba et al., 2011). In wild-type MEFs, the dissipation of such potential caused a deficiency in MAVS-mediated antiviral response. These results demonstrated that the ability of mitochondria to fuse correctly and to maintain an appropriate membrane potential are crucial events of MAVS signalling (Koshiba et al., 2011).

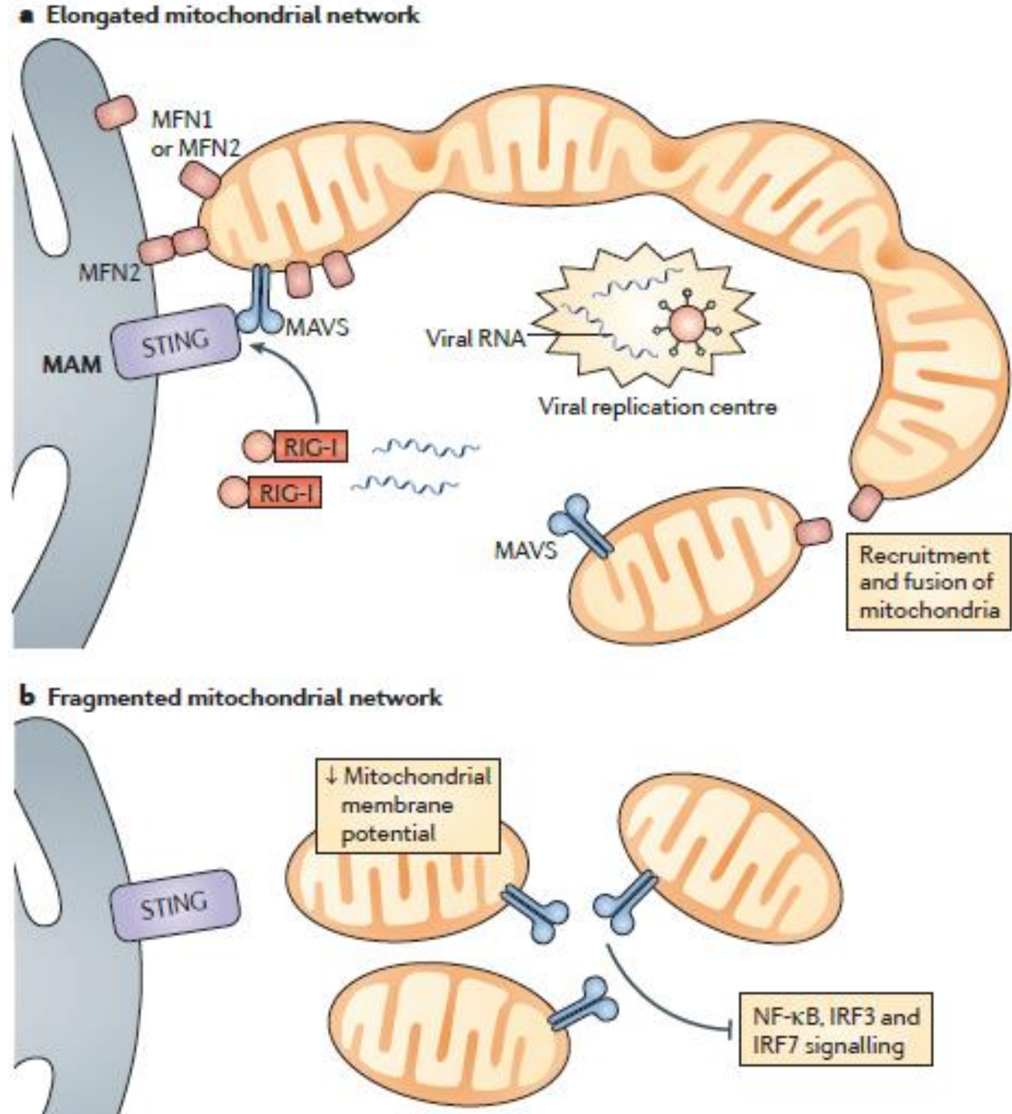

Figure 20 – Mitochondrial dynamics regulate MAVS signalling.
a) During infection, RIG-I and MAVS-enriched mitochondria are recruited around centres of viral replication to promote MAVS signalling. This occurs as mitofusin 1 (MFN1) and MFN2 induce fusion of the mitochondrial network, which also serves to increase MAVS interactions with downstream signalling molecules. Mitochondrial MFN1 and MFN2 also interact with endoplasmic reticulum-localized MFN2, which promotes interactions between MAVS and STING at mitochondrial-associated membranes (MAMs). b) Fragmentation of the mitochondrial network – which is induced by viral infection, mitofusin deficiency or overexpression of fission-promoting molecules – results in decreased mitochondrial membrane potential and blocks interactions between MAVS and signalling molecules such as STING. This leads to reduced signalling by NF-kB, IRF3 and IRF7. (West et al., 2011)

Besides regulating mitochondrial fusion, **MFN2** is also important in the tethering between mitochondria and the endoplasmic reticulum (ER) at MAMs (mitochondrial associated membranes) (Horner et al., 2011). MAMs are a subcellular domain emerging in MAVS signalling. For instance, infected cells exhibit increased numbers of ER-elongated mitochondria contacts, in comparison with non-infected cells, suggesting that these contacts are augmented under mitochondrial fusion and elongation, induced

by infection. This is particularly important, given that the MAVS population that resides in MAMs is quite important for antiviral signalling (Horner et al., 2011). A recent study described a role for **FAK** (focal adhesion kinase) in positive regulation of MAVS-mediated antiviral signalling (Bozym et al., 2012). FAK is a tyrosine kinase protein that localizes in contact points between extracellular matrix and the intracellular cytoskeleton, known as focal adhesions. This study demonstrated that FAK-knockdown MEFs were highly susceptible to viral RNA but did not reveal susceptibility to DNA virus infections. FAK-knockdown MEFs were attenuated in IFN-β and NF-kB signalling in response to poly I:C. Surprisingly, co-immunoprecipitation and immunofluorescence microscopy assays demonstrated that FAK re-localizes from focal adhesions to the mitochondrial membrane, where it interacts with MAVS, in a viral infection-dependent manner. Despite of the exact role of FAK in MAVS signalling remain undetermined, MAVS and mitochondria distribution is quite abnormal in FAK-deficient cells, suggesting a potential role in mitochondrial dynamics regulation, required to facilitate MAVS downstream signalling (Bozym et al., 2012).

As previously mentioned in this chapter, **STING**, is an ER-linked protein that modulates MAVS signalling, and, in addition to its localization at the ER outer membrane in four transmembrane domains, STING is also expressed at the mitochondrial outer membrane (Zhong et al., 2008). Following RIG-I activation, STING interacts with MAVS and facilitates TBK1 recruitment and subsequent IRF3 phosphorylation (Zhong et al., 2008). STING is able to bind itself to RIG-I but not to MDA5 and does not respond to poly I:C activation, indicating that STING acts strictly downstream of RIG-I (Ishikawa and Barber, 2008). Interestingly, STING was shown to interact with components of the translocon-associated protein complex (TRAPβ) and SEC61β translocon, essential interactions for a proper STING signalling (Ishikawa and Barber, 2008). Taken together, these observations suggest that special regulation of STING at ER and mitochondria is essential for the RIG-I–dependent antiviral response, despite the mitochondrial and ER STING proportions remain difficult to determine, due to the close interaction between these two organelles. Cumulative evidences underline the importance of the physical platform – denominated Mitochondria associated membranes (MAMs) – that connect ER to mitochondria (Horner et al., 2011; Pinton et al., 2011). Disruption of MAMs has been linked to imbalances in calcium signalling, cellular stress and apoptosis deregulation (Pinton et al., 2011).

Regulation of **ROS** (reactive oxygen species) has also been reported to have a role in positive regulation of RLR signalling from mitochondria. Examining the mechanism for this phenomenon, *Zhao et al.* described **COX5B** (cytochrome C oxidase 5B) as a novel MAVS interacting partner, responsible for ROS repression and RLR signalling (Y. Zhao et al., 2012). COX5B is a mitochondrial protein and a member of the oxidase cytochrome c – known to catalyse the last step of the electron transport chain. Overexpression of COX5B diminished MAVS-mediated signalling without any effect in TLR or TNF-α mediated signals, suggesting an effect specific to the RLR pathway, while cells with COX5B depletion exhibit augmented antiviral signalling. Interestingly, in addition to its roles in ATP production, COX5B has also been shown to be involved in negative regulation of ROS production. In order to investigate the possible role of this pathway in MAVS regulation, the authors used two components known to alter ROS levels and found that an increase in ROS results in an increased MAVS signalling and diminished levels of ROS have the opposite effect. Additionally, cells expressing exogenous MAVS produced higher levels of ROS, which was disrupted by introduction of exogenous COX5B (Y. Zhao et al., 2012).

The autophagosome is another organelle involved in the regulation of the innate immune response. As mentioned before, the **Atg5-Atg12 conjugate** (essential for the formation of the autophagosome) was shown to negatively regulate RIG-I pathway by direct interactions with RIG-I and MAVS (Jounai et al., 2007). Additionally, IFN production was increased in response to poly I:C treatment in Atg5 knockout, autophagy-deficient cells, that are characterized for mitochondrial accumulation (Tal et al., 2009). This phenotype can be explained by the fact that autophagosomes are responsible for the removal of several types of old or damaged organelles, including mitochondria. Mitochondria accumulation has two effects: it leads to an increase of MAVS, what partially explains the increase in IFN production; and the accumulation of damaged mitochondria induces an increase in mitochondrial reactive oxygen species (mROS). The same study demonstrated that mROS can stimulate RLRs, ultimately leading to IFN induction (Tal et al., 2009).

2.4 – MAVS downstream signaling cascade: the antiviral effector response – type I IFN induction and ISG production

As previously stated at chapter 2.1, IRF3/IRF7 and NF-kB, once properly activated, translocate to the nucleus where they interact with the IFN-β promoter in a highly coordinated manner. The final steps from that point on, ending with the actual production of ISGs will now be explored in greater detail. Since IFN-α induction also depends upon IRFs actions, it will also be addressed in this chapter.

2.4.1 – IRF3 and IRF7 – the antiviral IRFs

The specificity of type I IFN induction is achieved by members of the transcription factor family, the Interferon Regulatory Factors (IRFs) (Honda et al., 2006; Nguyen et al., 1997). At total, nine interferon regulatory factors were identified: IRF-1, IRF-2, IRF-3, IRF-4/Pip/ICSAT, IRF-5, IRF-6, IRF-7, IRF-8/ICSBP and IRF-9/ISGF3g/p48 (Hiscott, 2007a; Nguyen et al., 1997). Each member shares extensive homology at their N-terminus DNA binding domain (DBD), characterized by five tryptophan repetition elements in their first 150 amino acids (Nguyen et al., 1997). The IRF DNA-binding domain mediates the specific binding to IFN-stimulated regulatory elements (ISREs) in IFN stimulated genes.(Honda et al., 2006; Nguyen et al., 1997)

In addition to their role in immunity, IRFs are also involved in the regulation of the cell cycle, apoptosis and tumour suppression (see *Figure 13*) (Honda et al., 2006). Each IRF contains an unique C-terminal, named IRF-association domain (IAD) (Honda et al., 2006; Nguyen et al., 1997). The particular function of a certain IRF its accounted by the IAD ability to interact with other members of the IRF family, through its transactivation potential, interaction with other factors, and the specific expression of IRFs in a given cell line (Hiscott, 2007a; Nguyen et al., 1997).

Table 2 - The IRF family members and their immune actions.
(Richez et al., 2010)

IRF	Role in immune system function and development
IRF1	Binds to the adapter MyD88 and, in cells pretreated with IFN-γ, increases the induction of TLR-dependent genes. Involved in IFN-β production by murine myeloid dendritic cells. Promotes Th1 differentiation
IRF2	Blunts the response to type I IFNs by antagonizing IRF1 and IRF9. Stimulates the expression of genes induced by IFN-γ in collaboration with IRF1. Required for the differentiation of CD4+ dendritic cells
IRF3	Enables the production of type I IFNs (IFN-α4 and IFN-β) and chemokines (CXCL10)
IRF4	Binds to MyD88 and inhibits the production of proinflammatory cytokines induced by TLR stimulation. Required for Th2 differentiation (IL-4) and Th17 differentiation. Supports B-cell development and differentiation to plasma cells
IRF5	Binds to MyD88 and TRIF and increases the production of proinflammatory cytokines and type I IFNs after TLR stimulation or viral infection. Suppresses oncogene-induced transformation. Required for apoptosis in response to DNA damage
IRF6	Required for keratinocyte differentiation
IRF7	Binds to MyD88 and TRIF and increases the production of type I IFNs induced by the stimulation of some TLRs
IRF8	Enables the production of type I IFNs in dendritic cells. Stimulates the production of IFN-γ. Required for the differentiation of CD8a+ dendritic cells and plasmacytoid dendritic cells. Supports B-cell development in association with IRF4. Enables Th1 differentiation via macrophages and dendritic cells
IRF9	Binds to STAT1 and STAT2, thereby forming ISGF3, which stimulates the genes induced by type I IFNs

IFN: interferon; IRF: interferon regulatory factor; TLR: toll-like receptor.

The search for IRFs that might activate IFN-α and IFN-β gene promoters led to the identification of IRF3 and IRF7, the so called “antiviral IRFs” (Au et al., 1995). The identification of these IRFs and their role in the transcriptional activation of type I IFN genes had a great impact in the comprehension of the molecular mechanisms of antiviral response induced by pathogens (Hemmi et al., 2004). It became quite obvious that, in spite of the recognition of pathogens being mediated by distinct cellular receptors and signalling pathways, all of them lead to IRF3/IRF7 activation, a critical step for the transcriptional activation of type I INF genes (Kishore et al., 2002; Paun, A; Pitha, 2007).

The IRF3 member was first identified in a database search for IRF1 and IRF2 homologues (Au et al., 1995). The IRF3 gene (single gene copy at chromosome 19q13.3-13.4) encodes a 427 aa. phosphoprotein of 55kDa, which is constitutively expressed throughout a majority of tissues and cell lines, sharing most homology with IRF-8 and IRF-9 (Bellingham et al., 1998; Lin et al., 1999; Mamane et al., 1999). The transcriptional activity of IRF3 is controlled by viruses, induced by dsRNA, phosphorylation events at Ser. 385 and Ser.386 at the C-terminal, as well as phosphorylation at a serine/threonine cluster between aa. 396 to 405 – mediated by IKK-related kinases (TBK1 and IKKε) (Lin et al., 1998; Sharma et al., 2003; Yoneyama et al., 1998). A model for IRF3 activation was proposed, where C-terminus phosphorylation induces a conformational change in IRF3 that allows homo and

heterodimerization, dependent of association with CREB-binding protein/p300 (CBP/p300) (Sharma et al., 2003; Weaver et al., 1998). The activated IRF3 translocates to the nucleus, while its association with CBP/p300 retains IRF3 inside the nucleus, facilitating IFN-β transcription (Weaver et al., 1998). The IRF3 phosphorylation, ultimately leads to its degradation via ubiquitin-proteasome (Hiscott, 2007b; Lin et al., 1998).

The IRF7 member was first described by its binding and repression functions at the Qp promoter of the Epstein Barr virus (EBV), which regulates the EBV antigen 1 (EBVA1) – that contains a ISRE-like element – but its relevance in IFN-α gene regulation was rapidly recognized (Au, 1998; Marié et al., 1998; Sato, Hata, et al., 1998). Unlike IRF3, IRF7 is not constitutively expressed (except for B cells and dendritic cells); instead, it is induced by viral infection, IFNs and it presents an half-life of only 30 minutes, which can represent a mechanism of transient induction of IFNs (Lin et al., 2000; Marié et al., 1998). Phosphorylation of serine residues acts as a catalyser for IRF7 activation and it is critical for its translocation to the nucleus; particularly, the C-terminus region between aa. 471-487 has been identified as the target for phosphorylation (Caillaud and Hovanessian, 2005).

Re-examining the RIG-I signaling pathway in *Figure 13*, it is clear that IRF3/IRF7 are the substrates of TBK-1 and IKKε kinases; both directly phosphorylate IRF3 and IRF7 at identical key residues at their respective C-terminals (Perry et al., 2004; Tenoever et al., 2007). The alignment of the primary sequence of the C-terminal domains of IRF3 and IRF7 revealed an extensive consensus motif SxSxxxS that appears to be the target of IKKε and TBK-1 (Hiscott, 2007a). Interestingly, RNAi treatment directly against IKKε and TBK1 reduces IRF3 phosphorylation and expression of IRF-dependent genes upon VSV infection in human cells (Benjamin et al., 2004). Further analysis of TBK1 -/- and IKKε -/- mice demonstrated that TBK1 is mainly involved in downstream signaling to IRF3 and IRF7 phosphorylation and development of an antiviral response, while recent experiments suggest that IKKε selectively regulates a subset of antiviral IFN genes during viral infection: IKKε-deficient mice produced normal levels of IFN-β but were hyper-susceptible to viral infection due to a defect in the IFN signaling pathway (Matsui et al., 2006; McWhirter et al., 2004). Specifically, a subset of IFN-stimulated genes wasn't activated in the absence of IKKε, once ISGF3 (Interferon-Stimulated Gene Factor 3) failed to bind to the promoters of the affected genes, thus confirming the requirement of the kinase activity of IKKε to regulate, at

least in part, the downstream transcription machinery necessary for the direct antiviral response (Tenoever et al., 2007).

2.4.2 – NF-kB & its antiviral role

The Rel/NF-kB family is the most studied collection of transcription factors. NF-kB proteins play their most evolutionarily conserved and relevant role at the immune system, regulating the expression of inducers and effectors in several points at the signaling pathways that define the response to pathogens. The range of NF-kB transcription factors, however, extends beyond the transcriptional regulation of immune responses, acting broadly to influence events of gene expression which have impact in cell survival, differentiation and proliferation (Hayden and Ghosh, 2008). Moreover, these transcription factors are found persistently active in a variety of disease states, including cancer, arthritis, chronic inflammation, asthma, neurodegenerative and heart diseases (Gilmore, 2014).

The Rel/NF-kB proteins are structurally related through an highly conserved domain, responsible for DNA binding and homo/hetero-dimerization, known as Rel homology domain (RHD). Nevertheless, Rel/NF-kB proteins can be organized in two categories, based on their sequences: C-terminus sequences to RH domains. The members of the first class – p105, p100 and *Drosophila Relish* – present long C-terminal domains, which contain multiple copies of ankyrin repeats; members of this class become active by limited proteolysis or disrupted translation (p105 to p50 and p100 to p52) (Gilmore, 2014). As a result, members of this class cannot act as transcription activators unless they dimerize with members of the second class, or with other proteins capable of recruiting co-activators (Hayden and Ghosh, 2008). The second class (the Rel proteins) include c-Rel, RelB, RelA (p65), *Drosophila Dorsal* and Dif, containing C-terminal transcription activation domains (TAD) (Gilmore, 2014).

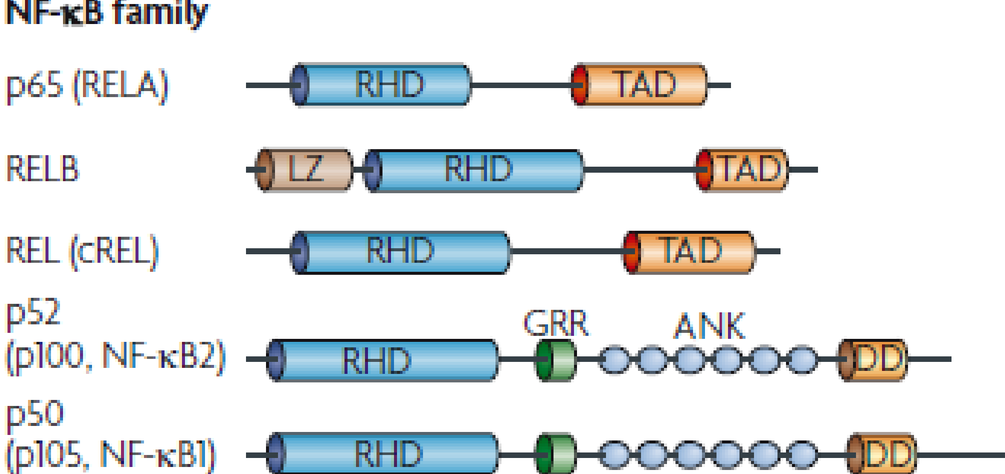

Figure 21 – The NF-kB family members and their structural features.
The main feature of the NF-kB family members is the presence of a Rel Homology Domain (RHD). The presence or absence of a Transactivation Domain (TAD) separates the family members in two categories. The precursor proteins p100 and p105 function as both, inhibitors of nuclear factor-κB and, when processed by the proteasome, become NF-κB members. RHD – Rel Homology Domain; TAD – Transactivation Domain; LZ – leucine zipper domain; GRR – glycine-rich region; DD – death domain. Adapted from: (Ghosh and Hayden, 2008; Hayden and Ghosh, 2008)

The Rel/NF-kB transcription factors bind to DNA sites of 9-10 base pairs – named kB sites – in the form of dimers. All vertebrate Rel proteins can form either homo or heterodimers, except RelB that can only form heterodimers (Gilmore, 2014). Particularly, the constitutive binding of p50 or p52 to kB sites at NF-kB-responsive promoters can act in order to verify NF-kB transactivation until they are dislocated by transcriptionally competent NF-kB dimers (Hayden and Ghosh, 2008). The combinatory diversity of homo and heterodimers that NF-kB proteins can form between them contributes to the regulation of distinct, but overlapping sets of genes, in which, individual dimers possess different specificities to DNA-binding sites in specific, but related, kB sites (Gilmore, 2014). Among the NF-kB dimers, the p50/p65 heterodimer is the biggest and most avidly forming Rel/NF-kB complex in most cells. Therefore, the NF-kB term will hereinafter be used referring to this particular heterodimer while Rel/NF-kB term will be used as a generic term, not referring to any particular dimer.

In their inactive state, Rel/NF-kB dimers are found associated with one of the three typical IκB proteins IκBα, IκBβ or IkBε or to the precursor proteins p100 and p105, maintaining Rel/NF-kB dimers predominately “trapped” at the cytoplasm, thus acting as the main regulators of their activity. All IκB proteins are characterized by the presence

of multiple domains with ankyrin repeats. The prototypical and most studied member of this family is IκBα. IκBα is rapidly degraded during activation of Rel/NF-kB, leading to the release of several Rel/NF-kB dimers, despite the fact that p50/p65 is the most likely target of IκBα. The established model of IκB postulates that IκBα retains NF-kB dimers at the cytoplasm, thus preventing nuclear translocation and subsequent DNA binding. In fact, the crystal structure of IκBα bound to p50/p65 heterodimer reveals that IκBα protein masks only a nuclear localization sequence (NLS) of p65, while the NLS of p50 remains exposed. The exposed NLS of p50 coupled with the nuclear export sequences (NES) of IκBα and p65 lead to a constant shuttling/redistribution of the IκBα/NF-kB complexes between the nucleus and cytoplasm, despite the steady-state location appearing to be almost exclusively cytosolic. On the other hand, degradation of IκBα (by phosphorylation events through IKK kinases) drastically alters the dynamic balance between cytosolic and nuclear signals, favouring the nuclear localization of the NF-kB dimers, thus allowing the transcriptional competent NF-kB dimers to bind to DNA at the previously mentioned kB sites. In the case of the repression of Rel/NF-kB dimers by p100, proteasomal processing of p100 to p52 takes place (instead of degradation), consequently releasing Rel/NF-kB dimers containing p52, which leads to a transcriptional response distinct from the one induced by IκBα - in part due to the fact that IκBα degradation and p100 processing regulate different populations of Rel/NF-kB dimers, therefore regulating distinct sets of genes (Hayden and Ghosh, 2008).

Evoking once more the schematic representation of RIG-I signaling pathway depicted in *Figure 13* it is discernible that, while the IRF3/IRF7 activation takes place through the actions of IKKε and TBK1, the removal of the IκBα repression from NF-kB cytosolic dimers occurs due to a different kinase complex: two highly homologue kinase subunits – IKKα and IKKβ – and NEMO, the regulatory subunit. In the majority of the classic NF-kB pathways (such as the one induced by RIG-I signaling), IKKβ is necessary and sufficient to phosphorylate IκBα at Ser32 and Ser36; IKKα can also mediate IκBα phosphorylation and cause its degradation, but it is not a necessary occurring step. The alternative/non-canonical pathways rely only on the IKKα subunit, which phosphorylates p100 causing the inducible processing to p52. It should be noted that the alternative pathway is activated by a subset of the Tumour Necrosis Factor Receptor (TNFR) superfamily while the canonical pathway is activated by a broader and overlapping set of receptors. The phosphorylation of serine residues at IκB proteins results in their K48-linked polyubiquitination by SCFβTrCP ubiquitin ligase complex in coordination with E2 UbcH5. The newly released NF-kB dimers can then bind to enhancer and promoter kB sites at the DNA sequence, allowing the interferon

response to take place. The termination of NF-kB response depends on the transcription of new IκB molecules that will interact with the competent NF-kB proteins bound to DNA sequences, trapping them once again mainly at the cytoplasm (Hayden and Ghosh, 2008).

2.4.3 – IRF3/IRF7 & NF-kB recruitment and the assembly of the IFN-β "enhanceosome"

Upon viral infection, the host cell requires three transcription factors in order to activate the transcription of the IFN-β gene: NF-kB, IRF3/IRF7 and ATF-2/c-Jun (Ford and Thanos, 2010; Honda et al., 2005). These necessary proteins to IFN-β induction are present in the cell prior to viral infection, but only upon viral infection they become activate to induce an adequate antiviral response (Honda et al., 2005). IRF3/IRF7 and NF-kB activation was already described above, but, unlike NF-kB and IRF-3/IRF-7, ATF-2/c-Jun (Activating transcription factor 2/c-Jun) is found in the nucleus and it is not able to activate transcription until its activation domain is phosphorylated by a MAPK signalling pathway [MAPK signalling pathway can be reviewed at (Jeffrey et al., 2007; Munshi and Ramesh, 2013)]. ATF-2 and c-Jun belong to the Fos-Jun family of bZIP proteins that possess a basic DNA-binding domain and a coiled-coil leucine zipper dimerization domain (Chinenov and Kerppola, 2001). It is important to note that ATF-2/c-Jun requires the cooperative interactions with the other IFN-β activators to form a stable complex with DNA (Ford and Thanos, 2010).

Interestingly, the oxidative state of the host cell also seems to be important for proper IFN-β transcription activation (Ford and Thanos, 2010). Specifically, IRF3 is S-glutathionylated in non-infected cells but when a viral infection takes place, IRF3 is deglutathionylated by glutaredoxin-1 (GRX-1) (Prinarakis et al., 2008). The removal of glutathione moiety of IRF3 is necessary for the efficient interaction between IRF3 and its transcriptional co-activator CBP/p300 (Prinarakis et al., 2008). Therefore, a particular viral infection induces specific signalling cascades that result in post-translational modifications (S-deglutathionylation and phosphorylation of IRF3/IRF7; ATF-2/c-Jun recruitment; removal of IkBα repression from NF-kB), necessary to the assembly of NF-kB, IRF3/IRF7 and ATF-2/c-Jun in a nucleic complex named "*enhanceosome*".

For a clear comprehension of how the enhanceosome is assembled, it is first crucial to understand the concept of nucleosome and to explore the characteristics of the IFN-β enhancer regions.

Nucleosomes are the basic units of eukaryotic chromatin, composed of an histone octamer around which ~147bp of DNA is wrapped (Ford and Thanos, 2010). Each histone is made of two copies of histone proteins H2A, H2B, H3 and H4; histone tails at N-terminus extend from the central structure and are subject of covalent modifications like acetylation, phosphorylation and methylation (Kouzarides, 2007). These modifications are carried out by enzymes that are specifically recruited to the chromatin by regulatory transcriptional proteins and target the underlying genes for transcriptional activation or repression (Kouzarides, 2007). Histone modifications can also influence the nucleosome stability and provide an additional layer of information to the DNA sequence, in order to recruit additional regulatory transcription proteins (Bannister and Kouzarides, 2011; Kouzarides, 2007). The strategic nucleosome position throughout chromatin in selected genes can play a truly regulatory role in gene expression control; specifically, the phasing and the rotational or translational context of a given nucleosome can preferentially allow the binding of selected proteins to the adjacent DNA sequence, operating as a “gatekeeper” to access the DNA code (Schones et al., 2008).

The IFN-β enhancer is a DNA regulatory element of 50bp, localized between -104 and -55bp upstream of the transcription initiation site; it contains four positive regulatory domains (PRDs) named PRDII, PRDIII-I and PRDIV that bind NF-kB, IRFs and the ATF-2/c-Jun heterodimer, respectively (Ford and Thanos, 2010; Sato, Tanaka, et al., 1998; Visvanathan and Goodbourn, 1989; Wathelet et al., 1998). Together with a high mobility group protein A1 (HMGA1), these activators bind cooperatively to DNA and form an exceptionally stable complex designated enhanceosome – activating transcription from the IFN-β promoter, in response to a given viral infection (Thanos and Maniatis, 1995; Wathelet et al., 1998; Yie et al., 1999). None of these activators and DNA elements alone are capable of activating transcription, thus, there’s something special about this enhancer region that limits the transcriptional potential of its positive regulatory elements (Ford and Thanos, 2010). Analysing each PRD of the IFN-β enhancer region it is possible to understand how this highly regulated promoter, the enhanceosome, operates (Ford and Thanos, 2010).

PRDII site is recognized and bound by NF-kB transcription factor in the form of p50/p65 heterodimer with low affinity (Ford and Thanos, 2010). One of the reasons for

this low affinity seems to be an intrinsic bend in PRDII element that needs to be unfolded and aligned for a steadier NF-kB binding (Falvo et al., 1995). The chromatin-associated protein HMGA1 increases greatly the affinity of NF-kB to PRDII site (Falvo et al., 1995). HMGA1 is a chromatin architectural protein that alters DNA structure upon binding to the minor groove; specifically, in the case of PRDII, HMGA1 reverses the intrinsic bend in DNA and creates a more favourable surface for NF-kB binding – DNA-induced allostery (Falvo et al., 1995). Thereby, studies and assays isolating PRDII site and studying it in and out of its natural context, NF-kB and HMGA1 showed to facilitate transcription through their cooperative binding to PRDII at the IFN-β enhancer (Falvo et al., 1995; Ford and Thanos, 2010).

The PRDIV site of IFN-β enhancer binds an ATF-2/c-Jun heterodimer (Panne et al., 2004). As in the case of PRDII, PRDIV has an intrinsic bend of ~25° that is reduced to about 15° upon binding of HMGA1 (Falvo et al., 1995). The ATF-2/c-Jun binding reduces this bend in a way that the helix axis becomes almost straight, with some localized distortions required for a cooperative binding (Panne et al., 2004). Once again, HMGA1 binding proves to be essential for virus-induced activation of IFN-β gene (Falvo et al., 1995; Panne et al., 2004).

PRDIII-I site is the binding site of IRF3 and IRF7 proteins, yet, IRF1 was the first protein that was shown to bind to PRDIII-I (Ford and Thanos, 2010; Fujita et al., 1989). However, while IRF1 expression can indeed activate IFN-β transcription, it is not able to do it in a way that is consistent with virus activation alone (Reis et al., 1994). On the other hand, the IFN-β expression is severely deficient in mouse cells without IRF3 or IRF7, and it is pretty much undetectable in IRF3/IRF7 double knockout cells (Sato et al., 2000). Remarkably, IRF3 is constitutively expressed, phosphorylated upon viral infection and then dimerizes and translocates to the nucleus, while IRF7 is only expressed in low levels at first (Sato et al., 2000). Only after secretion of IFN-β, a signal cascade is induced to result in the expression of higher levels of IRF7 (Sato et al., 2000). Despite the differences between mouse and human cells, as well as the variability between cell lines, the bigger picture drawn by *in vitro* binding studies, transcription assays in human cells and knockout mouse models points to a model where high levels of IRF3 and low levels of IRF7 activate the initial IFN-β transcription that is then followed by a secondary stage with high levels of IRF7 expression, not requiring IRF3 (Reis et al., 1994; Sato et al., 2000).

2.4.4 – IFN-β enhanceosome assembly

Taking in consideration the data described above, several crystal structures of DNA-binding domains (DBDs) of proteins found in the enhanceosome have been resolved: the NF-kB p50/p65 heterodimer bound to PRDII, IRF1 bound to PRDI, IRF3 bound to PRDIII-I, ATF-2/c-Jun and IRF3 bound to a composite of PRDIV-PRDIII as well as p50/p65 and IRF7 bound to a composite of PRDI-PRDII (Berkowitz et al., 2002; Escalante et al., 1998; Panne et al., 2004, 2007; Qin et al., 2005). Using these structures and overlapping them, the entire enhanceosome, with the exception of HMGA1, has been deciphered, supporting the aforementioned biochemical models (Panne et al., 2007). In particular, ATF-2/c-Jun binds to PRDIV site with ATF-2 upstream and c-Jun downstream; four IRF molecules are bound to PRDIII-I site in the form of IRF3 homodimers or in the form of IRF3/IRF7 heterodimers – two IRF molecules bind as a dimer in one side of the DNA helix at the PRDI and PRDIII consensus sites and, at the opposite side of the helix, a second IRF dimer binds to non-consensus sites traversing the PRDI and PRDIII sites; and lastly, p50/p65 binds to PRDII site with p50 subunit upstream and p65 facing the central promoter (Panne et al., 2007).

An interesting feature to take into account during the enhanceosome assembly is the role that HMGA1 plays, since the crystal structures and biochemical data conflict at this point. HMGA1 binds to DNA at the minor groove through AT-hooks – each HMGA1 molecule contains three flexible AT-hooks DNA-binding domains (DBDs) (Reeves and Beckerbauer, 2001; Yie et al., 1997). An initial HMGA1 molecule binds to PRDIV site through its first and second DBDs, while a second HMGA1 binds to PRDII composite using its second and third DBDs (see *Figure 22*) (Yie et al., 1997). Curiously, the crystal structures show that one of the HMGA1 binding sites to PRDIV is obstructed by IRF7; at the PRDII site, p50/p65 binding distorts the DNA helix in a way that the minor groove becomes probably too narrow to accommodate a simultaneous binding to HMGA1 as well (Ford and Thanos, 2010). This could lead to the assumption that HMGA1 exerts its functions in a “hit and run” mechanism, that is, facilitating the enhanceosome assembly but not being present in the final structure (Ford and Thanos, 2010). However, HMGA1 was observed in the complete form of the enhanceosome, making direct contacts with NF-kB and ATF-2 (Yie et al., 1999). Besides, HMGA1 remains associated with IFN-β promoter from the time NF-kB is initially recruited till the end of IFN-β transcription (Ford and Thanos, 2010). Thus, it seems likely that the final

enhanceosome structure might be slightly different than the one that crystal resolutions have shown so far, or, perhaps, HMGA1 can be retained at the enhancer through protein-protein interactions (Ford and Thanos, 2010).

2.4.5 – Enhanceosome apparatus – the pre-initiation complex

The newly assembled enhanceosome creates a tree-dimensional surface that is initially recognized by the co-activator protein PCAF (p300/CBP-associated factor) (Agalioti et al., 2000). In spite of the fact that the IFN-β enhancer is directly flanked by nucleosomes in both sides, the enhancer sequence itself is nucleosome-free (Agalioti et al., 2000). Upon recruitment, PCAF acetylates a small subset of lysines at the histone tails of H3 and H4 at the adjacent nucleosomes, specifically the acetylation of H4K8 and H3K9 (Agalioti et al., 2002). This event is followed by phosphorylation of Ser10 at H3 by an unknown enzyme that, in turn, allows acetylation of H3K14 (Agalioti et al., 2002). In *in vitro* acetylation assays, PCAF reveals low substrate specificity, thus, the tree-dimensional configuration of the enhanceosome might position PCAF within this pre-initiation complex and convert it from a relatively unspecific enzyme into an enzyme with high levels of specificity – proving evidence of how an unique combination of activators can result in the generation of a very specific signal (Agalioti et al., 2002).

The combination of acetylation of histones is then recognized by a specific set of factors (Agalioti et al., 2002). At this point, PCAF is released and replaced by CREB-binding protein (CBP), which is recruited in the form of a complex with the holoenzyme RNA polymerase II (Pol II), containing Transcription Factor II E (TFIIE), TFIIH, TFIIF and SWI/SNF (SWItch/Sucrose Non Fermentable) (*Figure 22*) (Agalioti et al., 2002; Kim et al., 1998). Despite CBP being able to form a complex with each one of the activators of the enhanceosome alone, the tree-dimensional geometry creates a contiguous surface that facilitates cooperative binding of CBP with the enhanceosome, necessary for the synergic transcription of IFN-β (Merika et al., 1998).

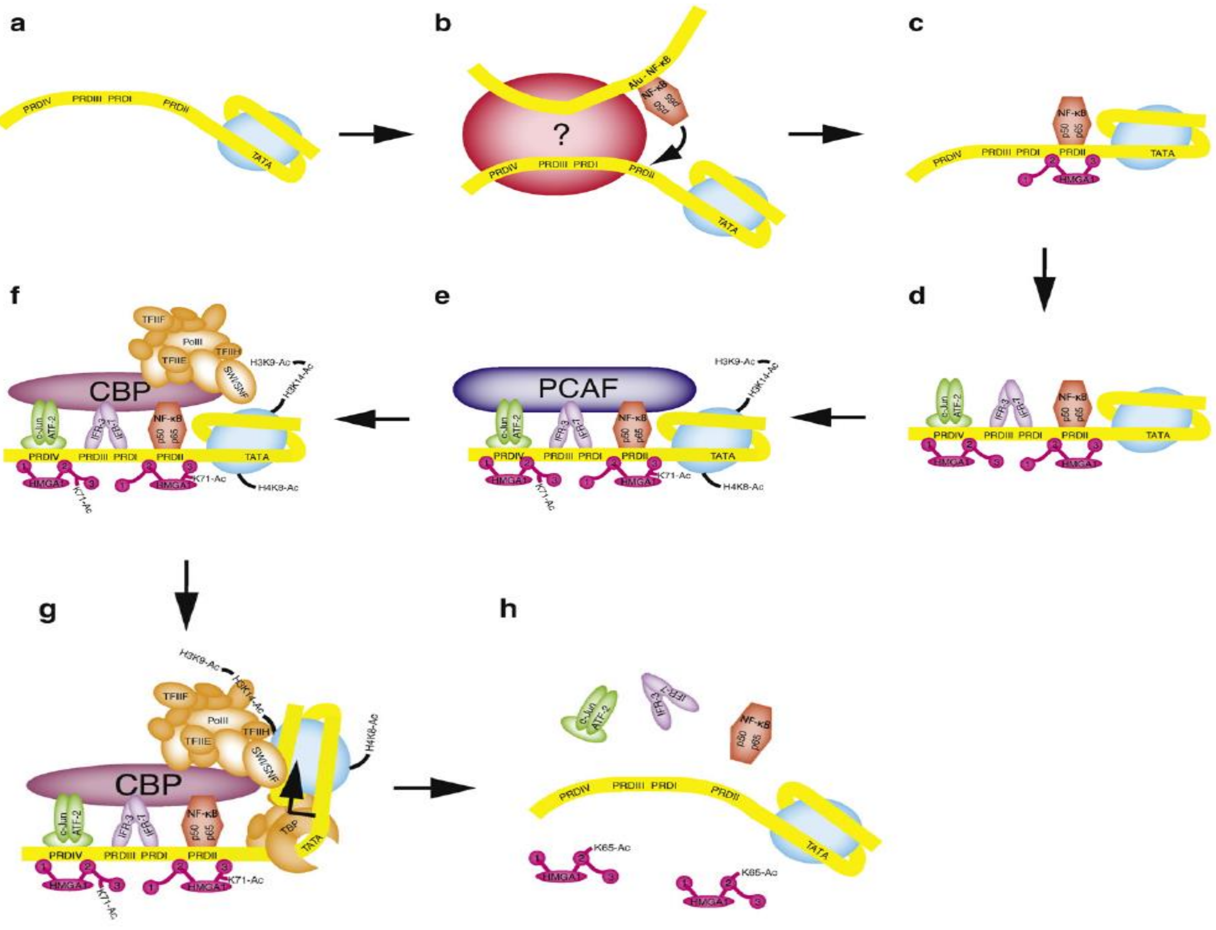

Figure 22 – Assembly of the IFN-β enhanceosome.
a) The naked enhancer DNA with its intrinsic curve. b) A specialized Alu element with a NF-kB binding site delivers NF-kB to the IFN-β enhancer. c) NF-kB binds to the IFN-β enhancer cooperatively with HMGA1 and reverses the intrinsic bend in the DNA at the PRDII site. d) ATF-2/c-Jun and IRF3/IRF7 bind in highly cooperative fashion along with NF-kB and HMGA1. e) PCAF is recruited to the enhancer and acetylates HMGA1 and histones H3 and H4. f) A CBP-PolII holoenzyme complex replaces PCAF, SWI/SNF remodels the adjacent nucleosome. g) TFIID (TBP) binds and induces nucleosome sliding. h) CBP acetylates HMGA1 and the enhanceosome proteins disassociate from the enhancer. (Ford and Thanos, 2010)

The IFN-β promoter has a nucleosome positioned on top of the transcription initiation site, directly adjacent to the TATA box (Agalioti et al., 2000). This is a common arrangement at the promoter that – together with the fact that transcriptional machinery cannot operate on nucleosome-bound DNA – underlines the importance of the local chromatin structure in the regulation of transcription (Agalioti et al., 2000). In order to overcome the barrier presented by the nucleosome positioned at the initiation site, the ATP-dependent nucleosome-remodelling SWI/SNF complex is recruited to the promoter through the interaction with CBP (Agalioti et al., 2000). The SWI/SNF BRG1 and BRM subunits possess bromodomains that specifically recognize the acetylated forms of the histone tails (Agalioti et al., 2000). The SWI/SNF complex promotes the

twisting and "peeling off" the DNA, in order to allow the binding of TFIID (Lomvardas and Thanos, 2001). Upon TFIID binding to DNA, a TATA-binding protein (TBP) induces a major DNA bending that causes the nucleosome to slide 36bp below its original position (Lomvardas and Thanos, 2001). With the TATA box now exposed, the IFN-β gene expression events can take place, culminating at this point in the production of interferon β proteins.

2.4.6 – The intricate regulation of IFN-α genes

The IFN-α genes are also transcriptionally induced in response to viral infections in a similar way to IFN-β, requiring serine-phosphorylated proteins for its expression (Sato et al., 2000). In some ways, IFN-α is less complex than IFN-β gene expression, since the only well-characterized enhancer elements that control IFN-α genes contain IRF-binding sites – with no apparent contribution of NF-kB or ATF-2/c-Jun complexes (Sato et al., 2000). The IFN-α genes negative regulation also appears to operate greatly through the inhibition of IRF proteins (Levy and Marié, 2005).

It is not yet clear if the IFN-α genes transcription involves the formation of an enhanceosome structure, DNA bending or nucleosome repositioning; it is, however, known that a competitive binding between IRF2 and activating members of IRF family keep the basal levels of IFNs, observed in the absence of viral infection (Levy and Marié, 2005). The repressor Pitx1 homeobox also seems to contribute to the silencing of IFN-α gene, through its ability to interact and inhibit IRF3 and IRF7 (Island and Mesplede, 2002).

Despite the deceptive simplicity of a regulatory scheme that only requires one type of transcriptional activator, there's a complexity aspect that is not observed with IFN-β (Levy and Marié, 2005). It was discovered that IFN-β and the multigenic family of IFN-α are not regulated in unison during a viral infection, and that their differential expression is, at least in part, regulated through a positive feedback loop involving the induction of IRF proteins (Marié et al., 1998; Sato, Hata, et al., 1998). IFN-β and the mouse IFN-α4 isotype are induced with early kinetics through the actions of the constitutively expressed IRF3 (Marié et al., 1998). On the other hand, enhancers from other members of the IFN-α family aren't able to bind IRF3, but instead, are only activated by IRF7 (Levy and Marié, 2005). IRF7, as previously stated in this chapter, is not constitutively expressed in most cell lines and its expression is induced by the IFN

signaling through the JAK-STAT pathway (Mamane et al., 1999). Thus, in response to the initial IFN-β and IFN-α4 secretion through the actions of IRF3, the IRF7 induction renders cells sensible to the induction of additional subtypes of IFN-α (commonly referred as the non-IFN-α4 subsets of genes), ultimately leading to a robust production of several species of IFN-α and a consequent powerful antiviral activity (Marié et al., 1998; Sato, Hata, et al., 1998).

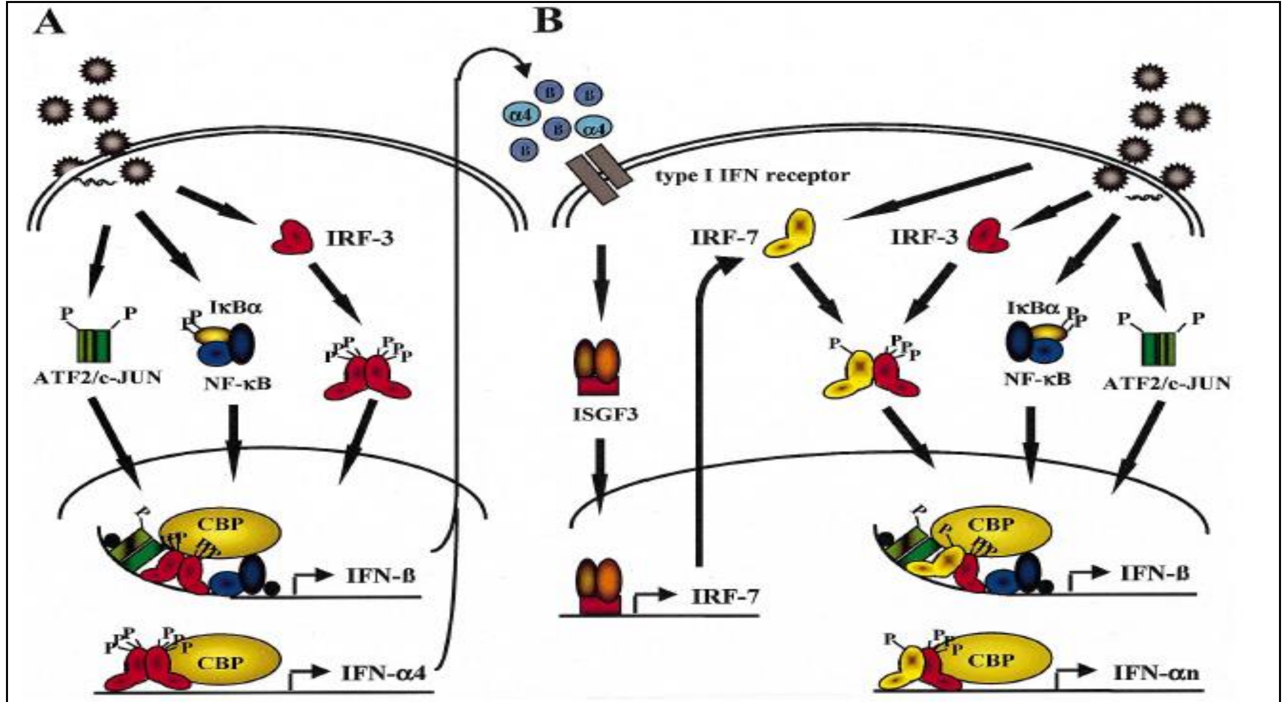

Figure 23 – Schematic model of the IFN gene induction in virus infected cells.
A) Activation of immediate-early genes: following virus infection or treatment with dsRNA, a coordinate activation of different transcription factors occurs through the activation of distinct signalling pathways that lead to virus-induced phosphorylation (P). These factors act synergistically at the IFN-β enhanceosome together with the co-activator CBP/p300 and the chromatin remodelling protein HMG1. In murine cells, activated IRF3 also upregulates IFN-α4 gene expression. B) Activation of delayed-type genes: secreted IFN from virus-infected cells acts in an autocrine or paracrine fashion through binding to the type I IFN receptor. Activation of the JAK-STAT signalling pathway induces the formation of the ISGF3 complex, which leads to the expression and activation of IRF7. In turn, IRF7 participates in the induction of delayed-type IFNs, resulting in the amplification of IFN gene expression. Transcribed from: (Mamane et al., 1999)

Additional reports have suggested another level of complexity in the regulation of IFN-α induction. In the same way that IRF3 and IRF7 presence and activation programs the induction of a diversity of IFN-α genes subtypes, other members of the IRF family seem to target specific IFN-α isotypes; particularly, it was found that IRF5 can participate in IFN-α genes induction by certain viruses, such as the Newcastle

Disease Virus (NDV), leading to the preferential induction of the human IFNA8 gene (Barnes et al., 2001).

2.4.7 – The IFN-dependent JAK-STAT pathway activation and ISG production

All interferons exert their effects by binding to specific surface receptors, leading to the activation of signalling pathways that ultimately target the induction of interferon-stimulated genes (ISGs), whose proteins mediate the antiviral effects (Fensterl and Sen, 2009).

Type I IFNs bind to a common surface receptor know as type I interferon receptor; it possesses two subunits, IFNAR1 and IFNAR2 and each one of these subunits interacts with a member of the Janus Activated Kinase (JAK) family. IFNAR1 is associated with tyrosine kinase 2 (TYK2) while IFNAR2 is associated with JAK1 (Platanias, 2005).

The binding of IFN-α or IFN-β to the type I IFN receptor results in the autophosphorylation and activation of the associated JAKs, TYK2 and JAK1, that, in turn, regulate the phosphorylation and activation of Signal Transducers and Activators of Transcription (STATs). STATs are latent cytosolic transcription factors that become active upon phosphorylation by the Janus kinases. In response to type I IFN stimulation, activated STATs include STAT1, -2, 3 and -5. STAT4 and -6 can also be activated by IFN-α but only on certain cell types such as endothelial cells of lymphoid cells. Upon phosphorylation by JAKs, the activated STATs can form homo- or heterodimers that translocate to the nucleus and initiate transcription by binding to specific sites at the ISGs promoters (Platanias, 2005).

Worthy of note is the formation of the ISG factor 3 complex, composed of the active forms of STAT1 and STAT2, together with IRF9. This is an unique complex that binds to specific genic elements known as IFN-stimulated response elements (ISREs), presented at the promoter region of certain ISGs, thus initiating their transcription. Other types of complexes can bind to another type of element, known as Gamma interferon Activation Sites (GAS) in other ISGs promoter regions. This is particularly important, given that some ISGs only possess ISREs, others just the GAS and others can have both, thus, the combination of different STAT complexes allows a differential and optimal gene transcription (Platanias, 2005).

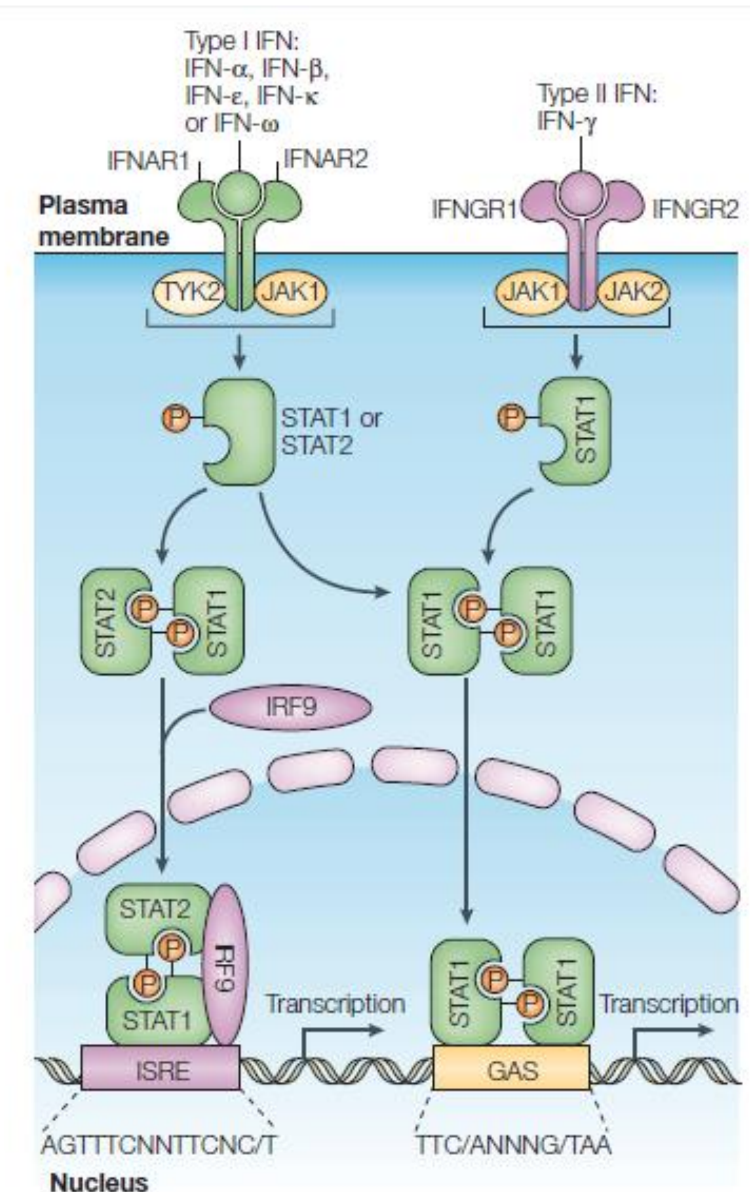

Figure 24 – Activation of classical JAK-STAT pathways through IFN receptors by type I and type II interferons.

All type I interferons bind a common receptor at the surface of human cells, which is known as the type I IFN receptor. This receptor is composed of two subunits, IFNAR1 and IFNAR2, which are associated with TYK2 and JAK1 respectively. The type II IFN, IFNγ, binds a distinct cell-surface receptor, known as the type II IFN receptor, which also has two subunits, IFNGR1 and IFNGR2 that are associated with JAK1 and JAK2 respectively. Activation of the JAKs that are associated with the type I IFN receptor results in tyrosine phosphorylation of STAT2 and STAT1, leading to the formation of STAT1-STAT2-IRF9 complexes, that are known as ISGF3 complexes. These complexes translocate to the nucleus and bind to ISREs in DNA to initiate gene transcription. Both, type I and type II IFNs also induce the formation of STAT1-STAT1 homodimers that translocate to the nucleus and bind GAS elements that are present in the promoter of certain ISGs, thereby initiating the transcription of these genes. N – any nucleotide. (Platanias, 2005)

The transcriptional activation of ISGs leads to the production of antiviral effector proteins. To date, three antiviral pathways were strongly established: the Protein

Kinase K (PKR), the 2-5 OAS/RNaseL system and the Mx proteins (Haller et al., 2006). However, other ISGs action mechanisms have been discovered in the past years, such as APOBEC3G, ISG15, IFIT1/2/3, viperin (also known as RSAD2), etc. (Schoggins and Rice, 2011).

The dsRNA-activated kinase protein **PKR** is induced by interferons but it is constitutively expressed in low levels in most cell types. Its activation occurs after binding of dsRNA inducing its dimerization and auto-phosphorylation. PKR then phosphorylates the translation initiator factor eIF-2α, resulting in its cellular and viral inhibition. PKR inhibits replication of most RNA viruses such as Vesicular stomatitis virus (VSV), Encephalomyocarditis virus (EMCV), West Nile virus (WNV), hepatitis C virus (HCV), and DNA viruses such as Herpes simplex virus 1 (HSV-1) (Fensterl and Sen, 2009).

Another type of IFN-induced enzymes is the **2'-5'-oligoadenilatesinthetases** (such as OAS1) that, upon binding with dsRNA generate 2'-5'-linked AMP oligomers from ATP; these activate RNase L which dimerizes and cleaves cellular and viral ssRNA, preventing protein expression and occasionally induces apoptosis. This system is the main effector against picornaviruses and influenza A viruses. Furthermore, cellular cleaved RNAs can amplify the interferon induction serving as ligands of RIG-I. The unspecific cleavage of ssRNA can also occur after ISG20 induction, a 3'-exoribonuclease that inhibits VSV viruses (Fensterl and Sen, 2009).

Mx1 (a.k.a. MxA) uses a peculiar mechanism by disrupting cell membranes around the nucleus, binding to viral nucleocapsids, thus inhibiting viral intracellular trafficking. It is the main feature against orthomyxoviruses such as influenza viruses and others such as measles virus, VSV, Hantavirus and Semliki Forest virus (Fensterl and Sen, 2009).

The nucleic acid-editing **APO-BEC3G** and **-3F** are deoxyxytidine deaminases that inhibit retroviruses, acting through the introduction of C or U mutations within the viral reverse DNA thus, directly interfering with reverse transcription (Fensterl and Sen, 2009).

Members of the human gene family **ISG56** (IFIT1) are highly induced by IFN, dsRNA or viral infection. Four members comprise this gene family, among them the IFIT1/ISG56, IFIT2/ISG54, IFIT4/ISG60 and IFIT5/ISG58. They all encode proteins with multiple tetratricopeptide motifs which are protein-protein interaction motifs that act in

combination. These proteins bind to eIF-3; the binding of P56 proteins to eIF-3 causes the inhibition of initiation of protein synthesis (Sen and Sarkar, 2007).

ISG15 is another human gene highly induced by IFN, dsRNA and viruses. It encodes an ubiquitin-like protein named P15 and, like ubiquitin, it binds to target proteins by isopeptide linkages between lysine chains of their targets. The functional consequences of ISG15ylation are still to be determined; a study revealed that NS1B protein of influenza B virus specifically blocks ISGylated proteins suggesting that this process allows the virus to evade the effect of ISG15. Another study shows that ISG15 is the protein that mediates IFN action against HIV-1 morphogenesis; IFNs inhibit the HIV-1 virion release without affecting protein synthesis and these effects can be mimicked by ectopic expression of ISG15 and its activating enzymes (Sen and Sarkar, 2007).

Viperin can interfere with the envelope budding process of cytomegaloviruses (HCMV), HCV and influenza by disrupting lipid rafts at the membrane; however, the precise action mechanism is yet to be determined (Fensterl and Sen, 2009).

The number of ISGs is endless and their action mechanisms remain elusive. This reveals a special feature of the antiviral immune system: it acts by coordinating the actions of several effector molecules with a less powerful effect instead of falling back on only a few powerful effectors. Inducing a range of weak effectors in detriment of upregulating a small group of powerful genes might in fact be preferable from the host perspective, once the latter could result in cellular toxicity (Schoggins and Rice, 2011).

3 – Viral strategies of survival – subverting key points in the antiviral RLR signalling pathway

The mechanisms employed by viruses to escape innate immune responses, particularly, the type I IFN induction system through detection by cytosolic receptors (the RLR signalling pathway) rely in the subversion of key aspects of such pathways. The most direct mechanism is to avoid initial detection, via mechanisms that interfere with viral recognition. Some viruses count on the circumvention of components of the signalling pathways which lead to IFN production and, lastly, others resort to mechanisms that inhibit IFN-induced antiviral effector proteins.

3.1 - Viral mechanisms that subvert viral recognition

Some viruses are able to hide or degrade their own genomes. For example, tick-borne encephalitis virus delays cellular signalling by sequestering their RNA molecules into membrane compartments at the cytosol, where they cannot be accessed or recognized by PRRs (Miorin et al., 2012); the Japanese Encephalitis virus (JEV) can also hide its dsRNA in intracellular membranes. Instead of hiding it, Lassa Fever virus uses the 3'-5'-exonuclease activity of its nucleoprotein to digest its own dsRNA (Hastie et al., 2011), while the C protein of the human parainfluenza type 1 is thought to regulate the production of its own RNA in order to prevent it from accumulating within the cell (Boonyaratanakornkit et al., 2011).

RLRs, like other viral detection receptors can be hampered by viruses:

- The human Respiratory Syncytial virus (RSV) N protein inhibits both RIG-I and MDA5 (Lifland et al., 2012) while the HIV protease decreases the cytosolic levels of RIG-I, targeting it to lysosomes (Solis et al., 2011);
- Arenavirus nucleoproteins and Z proteins of the New World Arenaviruses were shown to bind to RIG-I and inhibit subsequent downstream signalling (Borrow et al., 2010);
- Toscana virus-derived non-structural proteins (NSs) interact with RIG-I, leading to its proteasomal degradation (Gori-Savellini et al., 2013);
- V proteins of paramyxoviruses promote interactions between RIG-I and LGP2 (Childs et al., 2012);
- Several viral proteins (i.e.: Arterivirus NS2 (van Kasteren et al., 2012), Kaposi Sarcoma-associated herpesvirus ORF64 (Inn et al., 2011), etc.) target RIG-I

through deubiquitinating enzymes (DUBs) that remove K63-linked ubiquitin chains from RIG-I, preventing its interaction with MAVS adaptor.

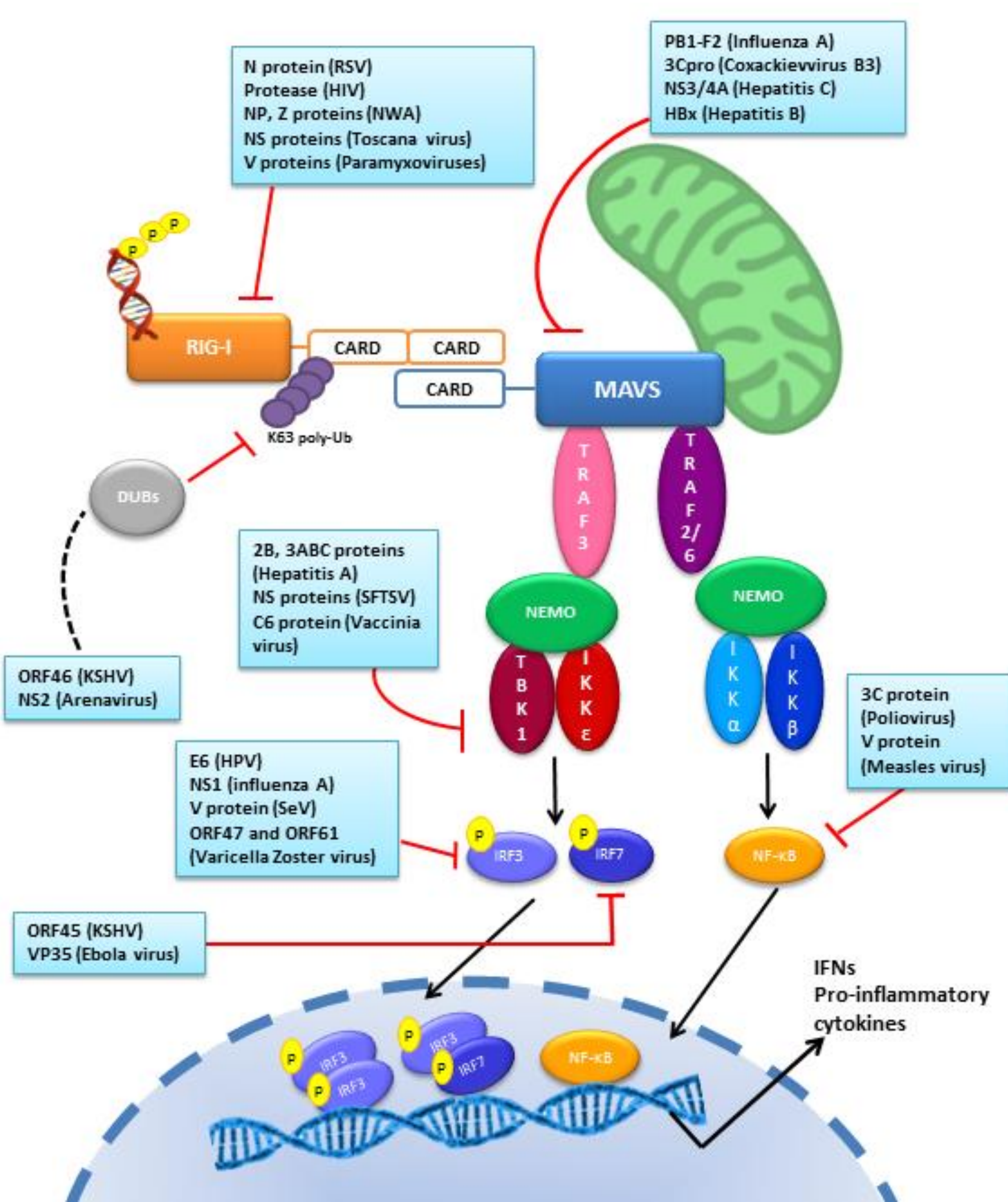

Figure 25 – The RLR pathway and viral inhibitors.
Several viral proteins/factors are capable of influencing and subverting the antiviral response by disrupting the RLR pathway in crucial points. DUBs – deubiquitinases; RSV - Respiratory Syncytial virus; HIV – Human Immunodeficiency virus; NWA - New World Arenaviruses; KSHV - Kaposi Sarcoma-associated herpesvirus; HPV – Human Papilloma virus; Sev – Sendai virus; SFTSV - Severe Fever with Thrombocytopenia Syndrome virus.

3.2 – Evasion mechanisms of RLR pathway components that lead to IFN production

MAVS adaptor is a common target for antiviral antagonists. PB1-F2 protein of influenza A virus binds to the transmembrane domain of MAVS leading to a drop in the mitochondrial membrane potential, required for MAVS functions (Varga et al., 2012). The coxsackievvirus B3 codes a cysteine protease 3Cpro and hepatitis C virus NS3/4A protein both cleave MAVS directly while HBx protein of hepatitis B virus associates with, and blocks MAVS (Kumar et al., 2011; Li et al., 2005; Mukherjee et al., 2011).

STING is also affected by viral proteins such as the protease complex NS2B3 of Dengue virus, cleaving STING in two inactive fragments (Yu et al., 2012). Papain-like proteases of human coronavirus NL63 and SARS-CoV that possess DUB and protease activities disrupt STING dimerization by increasing its ubiquitination levels (Sun et al., 2012).

It has been shown that IRF3 is a target for the viral protein E6 of human papilloma virus (HPV); *Ronco et al.* demonstrated that E6 interacts with IRF3 and compromises its ability to transcriptionally activate IFN-β production (Ronco et al., 1998). *Talon et al.* also demonstrated that influenza A prevents IRF3 activation by a mechanism dependent upon the viral protein NS1 (Talon et al., 2000). The V protein of Sendai virus binds directly to IRF3, jeopardizing its functions (Ye and Maniatis, 2011); Varicella zoster virus induces an atypical TBK1-independent IRF3 phosphorylation, blocking the downstream dimerization and its activity through the serine-threonine kinase protein ORF47 (Vandevenne et al., 2011); ORF61, also from varicella zoster virus, specifically interacts with activated and phosphorylated IRF3, using a RING-finger E3 ubiquitin ligase domain to ubiquitinate and degrade IRF3 via the proteasomal degradation (Zhu et al., 2011).

Several viral proteins are able to disrupt IRF3 activation via indirect mechanisms, by interfering with TBK1 and IKKε kinases. *Paulmann et al.* demonstrated that infection by hepatitis A virus inactivates TBK1 (Paulmann et al., 2008). TBK1 can also activate IRF7 inducing the production of IFN-α, and *Paulmann* group showed that virus-derived proteins 2B and 3ABC interact with MAVS and block TBK1 activity (Paulmann et al., 2008) Their findings also suggest an interaction between 2B and IKKε (Paulmann et al., 2008). Despite the exact mechanisms remaining elusive, the NS proteins of Severe Fever with Thrombocytopenia syndrome virus (SFTSV) and the HSV-1 y34.5 protein associate with and inhibit TBK1 (Ma et al., 2012; Qu et al., 2012). The A59 papain-like

protease domain 2 of the protein NSp3 of mouse hepatitis virus was found to deubiquitinate TBK1, decreasing its kinase activity, stabilizing it in an inactive conformation (Wang et al., 2011). The C6 protein of Vaccinia virus interferes with IRF3/IRF7 activation at the level of TBK1/IKKε through interaction with TANK, NAP1 and SINTBAD proteins (recalling that the contribution of these proteins to antiviral signalling is yet not clear) (Unterholzner et al., 2011). The NP proteins of several arenaviruses are capable of association with the kinase domain of IKKε, by encoding a miRNA known as miR-K12-11 that under regulates the mRNA translation of IKKε (Liang et al., 2011; Pythoud et al., 2012).

IRF7 can also be hampered by viruses: some strains of rotavirus use their NSP1 protein to cause IRF7 degradation via the proteasome, while other strains target IRF3 and IRF5, or even the β-TrCP protein, a component of the E3 ubiquitin ligase complex that activates NF-kB (Arnold and Patton, 2011). IRF7 is inhibited by competitive inhibition with ORF45 of Kaposi's sarcoma-associated herpesvirus (KSHV), which hinders IRF7 phosphorylation and activation, since it is more effectively phosphorylated by TBK1 and IKKε than IRF7 (Liang et al., 2012). ORF45 can also block IRF7 by association with its inhibitory domain, stabilizing auto-inhibitory interactions to keep the protein under a closed conformation (Sathish et al., 2011). In macrophages and dendritic cells, the VP35 protein of Ebola virus interferes with IRF7 activation through the RLR pathway while in plasmacytoid dendritic cells, VP35 does not block IFN production, since this cell type activates IRF7 through TLR pathway (Leung et al., 2011).

The KSHV express their own IRF proteins (vIRF) that possess a high homology with the cell IRFs; vIRF1 and vIRF2 of KSHV inhibit IRF3 and, subsequently impair IFN-β production (Devasthanam, 2014).

It has been reported that the 3C viral protein of poliovirus is able to cleave RelA subunit and functionally inactivate NF-kB in HeLa cells; RelA cleavage was also demonstrated for other viruses of the poliovirus family, suggesting that RelA cleavage can be a conserved characteristic within this virus family (Neznanov et al., 2005). Another example of viral disruption of NF-kB involves the V protein of measles virus that binds to the nuclear localization signal of RelA, compromising its nuclear translocation (Schuhmann et al., 2011). NEMO is also a target of cleavage, since its cleaved into inactive fragments by 3Cpro protease of foot-and-mouth disease virus (Wang, Fang, Li, et al., 2012).

Less understood is the viral action upon ATF2/c-Jun but a viral blockage was already described: the VP24 protein of Zaire Ebola virus prevents MAP kinase p38 phosphorylation (a precursor of ATF2 activation) and subsequent ATF2 activation (Halfmann et al., 2011).

3.3 – Inhibitory mechanisms of IFN downstream pathway components and IFN-induced effectors

Targeting the JAK-STAT pathway seems to be a commonly used strategy of viral subversion of the host attempts to fight viral replication. This is particularly true for a diverse group of viruses that express V and C proteins. The Paramyxovirus including measles, mumps and Hendra viruses express V proteins capable of blocking IFN-β induction. However, V proteins aren’t exclusive of paramyxoviruses: SV5 product of rubella virus has been shown to reduce the half-life of STAT proteins (Ulane and Horvath, 2002); V protein of Hendra virus is able to capture STAT proteins in large cytosolic complexes and limit their nuclear translocation (Rodriguez et al., 2003); measles-infected cells were found to be non-responsive to IFN-α but still responsive to IFNγ (Yokota et al., 2003); *Yokota et al.* investigated this and demonstrated that the V protein prevents the phosphorylation of JAK1 and the C protein binds to and incapacitates IFNAR1, preventing the IFN-α downstream events(Yokota et al., 2003).

Likewise, the C proteins also target the JAK-STAT pathway. C protein of Sendai virus was shown to inhibit STAT1 phosphorylation (Garcin and Marq, 2003); other studies also suggest that C protein of Sendai virus renders cells unresponsive to type I and type II interferons – *Gotoh et al.* found that C proteins interact with the phosphorylated forms of STAT1 and STAT2, reducing their ability to form homo- and heterodimers (Gotoh et al., 2003).

The human metapneumovirus reduces JAK1 and TYK2 mRNAs and proteins, leading to a diminished IFNAR expression by means of increased internalization, possibly due to the loss of TYK2 (Ren et al., 2011). E6 and E7 proteins of HPV interfere with STAT1 promoter to block its transcription (Hong et al., 2011); C protein of human parainfluenza virus type 1 prevents the nuclear translocation of STAT1, physically retain it at the cytosol in perinuclear aggregates associated with endosomal markers (Boonyaratanakornkit et al., 2011). vIRF2 of KSHV decreases STAT1 and IRF9 levels by compromising the functions of ISGF3 (Mutocheluh et al., 2011). HSV-2

causes the selective loss of STAT2 transcripts and proteins in some cell types while in others, the STAT2 levels remain constant by their phosphorylation and nuclear translocation are inhibited (Kadeppagari et al., 2012). Adenovirus on the other hand stabilizes the activated STAT1, capturing it in centres of viral replication, possibly through binding to viral DNA (Sohn and Hearing, 2011). Adenovirus also hampers dephosphorylation of STAT1 obstructing its interaction with the tyrosine phosphatase TC45 (Sohn and Hearing, 2011).

SOCS proteins are known for their ability to negatively regulate the JAK-STAT pathway. *Bode et al.* demonstrated in HepG2 cells that the core viral protein of HCV induces expression of SOCS3 (Bode et al., 2003). *Pothlichet et al.* revealed that, in respiratory epithelial cells infected with influenza A, the expression of SOCS1 and SOCS3 is induced (Pothlichet et al., 2008). Besides, expression of SOCS was only observed in signalling pathways were RIG-I and IFNAR1 were intact, suggesting that this mechanism has a late effect in the course of the infection (Pothlichet et al., 2008).

Fonseca et al. demonstrated that the antiviral activity requires mono-ubiquitination of histone 2B at lysine 120, a post-translational modification associated with transcriptionally activated chromatin in both, the ISG regions and their promoter regions (Fonseca et al., 2012). They also found that E1A protein of the human adenovirus disrupts the hBrel complex, responsible for the mono-ubiquitination of H2B, preventing ISGs expression and allowing the escape of the antiviral signalling (Fonseca et al., 2012). Another study by *Marazzi et al.* demonstrated that the NS1 protein of the H3N2 strain of influenza A virus has a short sequence that mimics the tail of the histone H3; this allows histone modification enzymes to act at NS1 protein (NS1 is acetylated and methylated in infected cells); the modified NS1 associates with the elongation complex of transcription PAF1, allowing the virus to sequester the elongation machinery (Marazzi et al., 2012). NS1 also disrupts transcriptional elongation in antiviral active genes, selectively jeopardizing ISGs expression (Marazzi et al., 2012).

Instead of globally obstructing the ISGs expression, some viruses target specific ISGs, such as the HCV infection that upregulates microRNA that decreases the expression of IFITM1 (Bhanja Chowdhury et al., 2012); ORF94 of HCMV blocks the expression and activity of OAS (Tan et al., 2011). Instead of interfering directly against OAS, Mouse Hepatitis Virus (MHV) uses NS2 protein, preventing the activation of RNaseL (L. Zhao et al., 2012). NS2A of JEV interacts physically with PKR to prevent its activation (Tu et al., 2012); poliovirus overcomes translational inhibition of PKR by

cleaving a eukaryotic initiation factor eIF5B, creating a cleavage fragment that is able to restore viral translation (White et al., 2011).

In particular cases, some viruses remarkably explore their host cell resources, using particular ISGs to their own advantage. In example, HSV-1 stimulates an isoform of MxA by alternative splicing in the absence of type I IFNs; this new isoform associates with components of the virion and compartments of nuclear viral replication, increasing viral replication (Ku et al., 2011). HCMV is long known for its ability to directly induce viperin expression in the absence of IFNs production (Seo, Yaneva, and Cresswell, 2011). It was recently demonstrated that, through interactions with the viral protein vMIA, viperin is relocated to mitochondria where it disrupts the actin cytoskeleton and increasing viral infection (Seo, Yaneva, Hinson, et al., 2011). Another curious example is the one of rotaviruses in intestinal epithelial cells, where it induces a strong induction of the type I IFN response, but, instead of limiting its growth, the IFN signalling promotes replication of rotavirus, especially in early stages (Frias et al., 2012).

4 – Peroxisome-dependent Antiviral Signalling – *via the MAVS adaptor*

Until recently, mitochondria were the single key organelle implicated in the RLR antiviral pathway. Notwithstanding, the results from *Dixit et al. (2010)* changed this perception, as they introduced an unexpected new player into this antiviral signaling pathway: the peroxisomes (Dixit et al., 2010).

4.1 - Discovery of the peroxisomal MAVS adaptor: first insights into this novel antiviral signalling platform

As aforementioned in the Introduction chapter, mitochondria and peroxisomes share several features and proteins (Schrader and Yoon, 2007). Among them, Mff and Fis1 share similarities in their sequence with the MAVS adaptor (Camões et al., 2009; Dixit et al., 2010) Exploring the implications of such similarities, *Dixit et al. 2010* reported for the first time that MAVS can also be found in peroxisomes.

The main findings of this study revealed the localization of MAVS proteins anchored to the membrane of peroxisomes and a functional signaling pathway that operates through peroxisomal MAVS. Such pathway establishes an immediate yet transient antiviral response when compared with the mitochondrial MAVS pathway. Furthermore, the authors suggest that this novel peroxisomal pathway might result in an ISG induction which is independent of type I interferons expression(Dixit et al., 2010).

The authors developed a strategy to distinguish the subcellular positioning of MAVS by generating different MAVS alleles that differ in their localization domain, so that they can study the isolated organelle-specific actions of MAVS and observe the different outcomes in the antiviral response. By deleting the MAVS localization motif, a MAVS-Cyto allele was created that was found not co-localize with any organelle, remaining in the cytosol; by replacing the localization motif of MAVS with the localization motif of peroxin Pex13, a MAVS-Pex allele was created and found to localize exclusively in peroxisomes. Two other MAVS alleles were generated by targeting two different mitochondrial proteins that reside in the mitochondrial outer membrane, OMP25 and Fis1. While the MAVS allele harbouring OMP25 was found to localize in both, mitochondria and peroxisomes (thus named MAVS-mimic since it mimics wild-type MAVS), the Fis1 allele selectively marked mitochondria, thus named MAVS-Mito (Dixit et al., 2010).

This system allowed the authors to establish that MAVS signaling occurs from both, mitochondria and peroxisomes, and that their actions seem to be complementary to each other. By examining the expression of a well-known ISG, viperin, in a model of reovirus infection, they observed the different outcomes in the antiviral response in cells harbouring the individualized MAVS alleles. In MAVS-Pex cells, viperin expression was shown to be rapid but, at the same time, transient, since viperin was present in early times of the infection, but its levels decrease over time. On the other hand, in MAVS-Mito cells, the viperin expression revealed delayed kinetics, with increasing and sustained viperin expression levels over time. This could easily indicate that, perhaps, the peroxisomal pathway establishes a first outburst of antiviral effector proteins to temporarily block the viral replication while the mitochondrial pathway, which takes more time to develop, is capable of inducing a stronger and longer lasting antiviral state to clear out the infection completely (Dixit et al., 2010).

These differences in the ISG induction over the course of a viral infection between peroxisome- and mitochondria-localized MAVS pathways prompted the authors to assume that more than one mechanism of ISG induction was taking place. As previously explained in chapter 2, the mitochondrial MAVS signaling pathway is known to induce type I IFN production that, in turn, induces ISG expression. So, as expected, MAVS-WT, MAVS-Mimic and MAVS-Mito cells triggered IFN production, but curiously, MAVS-Pex cells did not produce any detectable levels of IFNs. Thus, some kind of IFN-independent mechanism might be responsible to induce ISG production via the peroxisomal MAVS pathway (Dixit et al., 2010).

4.2 - Downstream effectors and correlation with IFN-independent ISG induction

Looking for some of the potential downstream regulators of the peroxisomal MAVS pathway, *Dixit et al. 2010* found TRAF3 and TRAF6 to be involved in this signaling pathway and some reporter genes for the functions of NF-kB and AP-1 were induced, as well as an IRF1 reporter and an ISRE that typically reports the activity of IRF3. Interestingly, the knockout of either IRF1 or IRF3 greatly compromised the ISG induction in MAVS-Pex cells, which might indicate a crucial role of these IRFs in the IFN-independent ISG induction via the peroxisomal MAVS pathway (Dixit et al., 2010).

These findings by seem to correlate with previous studies that attempted to explore the IFN-independent ISG induction pathway. Over the last decade it has been revealed that ISG production can actually be induced independently from IFN production, through the direct actions of IRF3, which is able to bind directly to the ISREs of the promoter regions of ISG genes. This happens in very early stages of the infection course and it's thought to be triggered solely by the entry of viral particles into the host cell (particularly, when dealing with enveloped viruses).

Paladino et al. 2006 came up with an hypothesis for the existence of an IFN-independent pathway to induce ISGs. For most infections, epithelia and fibroblasts are the first to contact with the virus, providing the first barrier of antiviral defence. Given the likelihood of these cells to be exposed to low levels of viral particles, epithelia and fibroblasts are prepared to respond to this stimulus by activating the constitutively expressed IRF3. Such response results in an IRF3-dependent, but IFN-independent, induction of a small subset of ISGs which are able to efficiently tackle the low viral load that entered the host cell. Subsequently, the host cell becomes efficient in controlling the infection within itself, without activating a more elaborate immune response. The main advantage of this early response is the prevention of the recruitment of immune cells and the secretion of pro-inflammatory cytokines that would most definitely cause unnecessary cell and tissue damage. *Paladino et al.* have also previously demonstrated the lack of IFNs and pro-inflammatory cytokines and then verified that the IFN-independent response is in fact specific for low levels of viral particles. However, if this first line of defence fails to control viral infection, the second line of defence comes into action with the production and secretion of IFNs, stimulating a more powerful and sustained immune response. The IFN production is crucial in this second phase, since it allows intercellular communication with the neighbouring cells in an attempt to prevent further viral spread (Paladino et al., 2006).

4.3 - Mitochondria and Peroxisomes: orchestrating an optimal antiviral response

Referring back to the *Dixit et al. 2010* study, they went on and analysed expression profiles in order to check if in fact the mitochondrial and peroxisomal antiviral responses are complementary and if they coincide with the general panel of expression of the wild-type MAVS cells. The profiles of MAVS-WT and MAVS-mimic were identical, as expected, and the profiles of MAVS-Pex and MAVS-Mito cells revealed a

transcriptome that is different from one another but that overlaps with the profile of MAVS-WT. This confirms that mitochondria and peroxisomes operate together to produce a maximal antiviral response. Furthermore, despite each organelle-specific pathway being capable to produce a functional antiviral response on their own, the integrated actions of both pathways produce a much greater and robust antiviral response – demonstrated by the fact that the magnitude of gene expression by MAVS-WT or MAVS-mimic was always greater than the ones in MAVS-Pex or MAVS-Mito individually (Dixit et al., 2010).

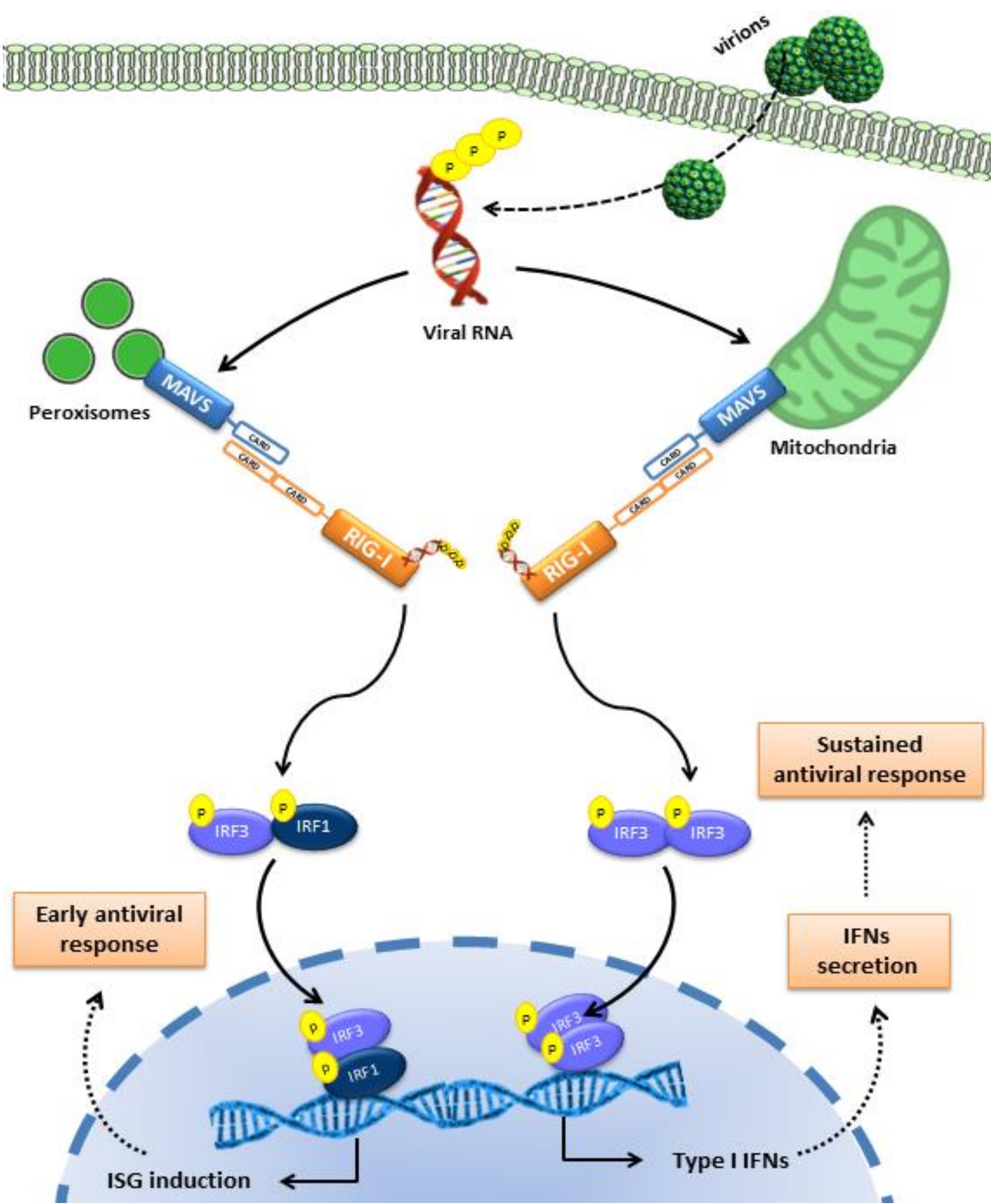

Figure 26 – Organelle-specific MAVS signalling.
The RNA helicases RIG-I or MDA5 detect viral RNA and then interact with MAVS on mitochondrial membranes. Detection of viral nucleic acids by these helicases and signalling via mitochondrial MAVS induces the expression of type I IFNs and eventually leads to virus control.

Dixit et al. 2010 show that MAVS is also present on peroxisomes. Peroxisomal MAVS is essential for the rapid expression of antiviral genes named ISGs. This gene expression, which is independent of type I IFN production, results in an early but transient antiviral response. However, type I IFN induction by the mitochondrial MAVS pathway is necessary for the eventual clearance of the virus. The different outcomes observed for the two MAVS pathways may relate to the fact that peroxisomal MAVS activates IRF1 in addition to IRF3. It is possible that an IRF1-IRF3 heterodimer induces expression of ISGs but not type I IFNS. Dixit et al. 2010 results reveal that MAVS signalling from both, peroxisomes and mitochondria, is necessary for maximal containment of virus replication. Text adapted from: (Sharma and Fitzgerald, 2010)

4.4 - The peroxisomal branch of the RLR pathway induces the expression of type III interferons

Despite the IFN-independent ISG induction being a plausible hypothesis for the peroxisome-dependent antiviral signalling pathway, – since it has been proven that several infections are capable of inducing the expression of ISGs independently from the induction of type I interferons – the *Kagan group* recently published a new manuscript that presents peroxisomes as a primary site for the initiation of the expression of type III interferons (Odendall et al., 2014).

This follow-up study started by trying to identify if MAVS-Pex cells were able to induce the secretion of any kind of extrinsic antiviral factors or if this pathway is entirely dependent upon cell-intrinsic factors, by evaluating STAT1 phosphorylation – a known downstream effector of the JAK-STAT pathway – which in turn is primarily stimulated by interferons. Interestingly, MAVS-Pex cells activated STAT1 phosphorylation, despite their inability to induce type I IFN expression. They established the requirement of STAT1 and the related JAK kinases (Jak1 and Jak2) in the peroxisome-dependent pathway with several inhibitors and small interfering (si)RNA-mediated knockdown studies, by determining their influence in the expression of viperin (Odendall et al., 2014).

In fact, they discovered that signalling from peroxisomes is dependent upon the actions of the JAK-STAT pathway, in order to create an antiviral cellular state. However, a key aspect of their findings was the involvement of Jak2 in the peroxisomal pathway, a factor that is not involved in type I IFN signalling. Thus, a valid candidate that has the properties of the factor secreted from peroxisomal MAVS which induces phosphorylation of Jak2 was found to be the recombinant human IFN-λ1, a type III interferon (Odendall et al., 2014).

They found out that an array of viruses such as reovirus, SeV and dengue virus as well as the bacterial pathogen *Listeria monocytogenes* induce the expression of type III

interferons via the RLR pathway in human cells. Their research went on to identify which MAVS-downstream components of the RLR pathway were responsible for the differential activation of the type I and type III interferon pathway, by evaluating the effects of IRF3, IRF7, NF-kB and AP-1 (the known requirements for the IFN-β enhanceosome). They discovered that IRF3 and NF-kB are required for the activation of the IFNL1 gene promoter (Odendall et al., 2014).

At this point, they focused their attention in a previous finding, the involvement of IRF1 in the peroxisomal-dependent antiviral pathway that was brought to light in their 2010 study. They now establish that, while the knockout of IRF1 has no influence in the IFNB1 mRNA levels (thus inferring no influence in the IFN-β expression), it completely abolishes the expression of IFNL1 mRNA after SeV infection, thus stating an important role of this transcription factor in the control of the expression of type III interferons (Odendall et al., 2014).

The results from these recent studies have revealed a fundamental gap in knowledge that involves the peroxisomal role in antiviral responses. Thus, future work is mandatory to enlighten the intricacies of the implicated signalling pathways that are in play.

5 – Discussion and Concluding Remarks

Taking into account the extensive bibliographic review presented in the previous chapters, it is very clear the gap in knowledge between the mitochondrial and peroxisomal antiviral pathways. It seems that the focus has been made in the search for a better understanding of the viral detection mechanisms – probably because PRRs are obvious targets for viral therapies or even the development of new vaccines.

Accordingly, it is still not clear the exact nature of MDA5 ligands, while RIG-I ligands properties that allow PRR detection were rapidly unveiled. The only characteristic that seems to make sense is the length of the RNA ligands, but that will likely not be the only feature that targets viral genomes to MDA5. Are there any other specificities that target viral RNAs to MDA5? And why do their ligands bind weaker to MDA5 than the ones that bind to RIG-I (in such way that it was even proposed that MDA5 might need the help of LGP2 to enhance the affinity of the long viral RNAs to MDA5)?

Besides the ligand-specificity aspect of MDA5 in the RLR pathway and, in light with the results of *Dixit et al., 2010,* another question arises: does MDA5, similarly to RIG-I, redirect the RLR pathway to peroxisomes? Are the outcomes of the viral infection similar to those induced by RIG-I interaction with MAVS? Some of these questions could be addressed using the MAVS mutant alleles strategy developed by *Dixit et al. 2010*, where MAVS-Mito and MAVS-Pex cells could be used to separately study the influence of the MAVS subcellular localization (mitochondria or peroxisomes) in the outcome of the antiviral response, MAVS-WT cells would be a viable model to test the antiviral outcome as a whole and MAVS-Cyto cells as a negative control. A possible co-localization between MDA5 and mitochondria or peroxisomes could be analysed by immunofluorescence microscopy using MDA5- and organelle-specific antibodies.

To isolate the MDA5-MAVS protein interactions, a co-immunoprecipitation of MDA5 with mitochondrial MAVS in MAVS-Mito cells or peroxisomal MAVS in MAVS-Pex cells could be performed. For the quantification of the MDA5-mediated signalling in the antiviral outcome in all types of MAVS alleles cells, ISG expression would have to be analysed by an Western blotting procedure, using a target for a specific ISG (i.e.: viperin, that's been shown to be induced in either MAVS-Mito and MAVS-Pex cells (Dixit et al., 2010)).

The contradicting role of LGP2 did not go unnoticed and has also been extensively pursued. It has been clarified that for certain types of viral infections it can exert positive regulatory roles (e.g.: encephalomyocarditis, vaccinia,and mengo viruses) while for others (e.g.: paramyxovirus Sendai, vesicular stomatitis, Newcastle disease, and influenza A viruses), it negatively regulates the RIG-I/MDA5 pathway (Zhu et al., 2014). Thus, it would be very interesting to study what stimulates LGP2 to exert a positive or negative role given a viral infection, or even if there is a particular feature to LGP2 or if there are any LGP2-interacting proteins that turns the regulatory role of LGP2 into a positive one or into a negative one.

By revealing that the subcellular localization of MAVS (mitochondria or peroxisomes) re-directs the downstream signalling pathways and influence the subsequent antiviral responses, *Dixit et al. 2010* shed a light into a novel aspect about signal transduction pathways: the compartment-specific signalling. Are there key elements in signalling pathways that rely on specific organelle localization in order to properly exert their functions? Can those pathways be re-directed to other organelles (where such key elements would be potentially located) and would they produce different responses? Those are some of the challenges worthy of being pursued in a near future.

The involvement of peroxisomes in immune responses might not have come as a total surprise, considering the fact that, mitochondria, the housekeeper of the cell and the most metabolic diverse organelle, participates in immunity, and shares several components and mechanisms with peroxisomes. However, the downstream signalling pathway implicating peroxisomes remains poorly studied.

Therefore, and based on the MAVS interacting partners in the well-established mitochondrial pathway, studies regarding the downstream components in the peroxisomal pathway are required. Such studies could be carried out in a model of MAVS-Pex cells infected with dsRNA viruses that are acknowledged to induce a powerful RIG-I activation (as it is the case of the reovirus or Sendai virus). Separate expression analysis would be performed for each and every component that is known to act downstream of mitochondrial MAVS (i.e.: TRAF2/6, TRAF3, NF-kB, IRF3, etc.) and, as controls for the level of expression of such components, these analyses would also have to be performed in infected MAVS-WT cells (positive control) and in non-infected cells (negative control). Quantification of the expression levels of every single component could be evaluated by Western Blot analysis.

Another aspect worthy of note regarding these two particular innate immunity signalling pathways is the discovery of their potential regulators. Most of the host regulators involved in the mitochondrial antiviral pathway were discussed in previous chapters and, as expected, the list kept growing over the years. Fluctuating between positive or negative regulation actions, a never ending array of molecules, proteins, etc. help to put together the complex puzzle of this pathway. Similar studies could be performed targeting the peroxisomal branch of the RLR signalling pathway, perhaps starting with some of the most well-known regulators of the mitochondrial branch.

The fact that viruses have developed strategies to evade, subvert and survive within the host, manipulating cellular machinery to its own advantage and replication, has been thoroughly investigated in several models of infection. For example, it was recently found that vMIA (an anti-apoptotic protein encoded by cytomegalovirus) which localizes at mitochondria, actually disrupts the MAVS signalling pathway by promoting mitochondrial fragmentation (Castanier and Arnoult, 2011; Poncet et al., 2006). Thus, it would be interesting to explore whether and how the peroxisomal MAVS signalling is a target of vMIA. Potential morphological changes in peroxisomes morphology (such as fragmentation and/or elongation) could be determined by immunostaining (in MAVS-Pex cells with previously transfected vMia) peroxisomes and vMIA through immunofluorescence microscopy.

Another example of a viral protein that directly influences the RLR pathway through mitochondrial MAVS is the hepatitis C virus ns3/4a protein, which has been shown to directly cleave MAVS, removing it from mitochondria (Li et al., 2005). The challenge would be to determine if ns3/4a also cleaves the peroxisomal MAVS and what are the effects on the expression of antiviral defence factors such as the ISGs. By overexpressing ns3/4a in MAVS-Pex cells and analysing it in different time points (through fluorescence microscopy) it could be determined if MAVS “loses” its peroxisomal localization and the cleavage could be further confirmed by immunoblotting the cleavage products. The implications in the outcome of an infection would be determined by quantification of the ISG production.

The possibilities are endless. Perhaps other organelles possess an immunological role, apart from their metabolic functions. As such, the study of host-pathogen interactions presents itself as a field with great potential for understanding, not only the implicated antiviral signalling pathways, but also the subcellular localization of such pathways and to unveil novel therapeutic targets that can be used for the development of new and more effective medical treatments.

7 - Bibliography

Adelberg, J. M. (2007). *Jawetz, Melnick, & Adelberg's Medical Microbiology*. (S. A. Brooks, Geo F.; Carroll, Karen C.; Butel, Janet S.; Morse, Ed.) (24th ed.). The McGraw-Hill Companies.

Agalioti, T., Chen, G., and Thanos, D. (2002). Deciphering the transcriptional histone acetylation code for a human gene. *Cell*, *111*(3), 381–92.

Agalioti, T., Lomvardas, S., Parekh, B., Yie, J., Maniatis, T., and Thanos, D. (2000). Ordered recruitment of chromatin modifying and general transcription factors to the IFN-beta promoter. *Cell*, *103*(4), 667–78.

Alberts, B. (2008). *Molecular Biology of the Cell. The Yale Journal of Biology and Medicine*.

Albrecht, T., Almond, J., Alfa, M., Alton, G., Aly, R., Asher, D., Baron, E., et al. (1996). *Medical Microbiology*. (S. Baron, Ed.) (4th ed.).

Allen, I., Moore, C., Schneider, M., and Lei, Y. (2011). NLRX1 protein attenuates inflammatory responses to infection by interfering with the RIG-I-MAVS and TRAF6-NF-κB signaling pathways. *Immunity*, *34*(6), 854–865. Elsevier Inc. doi: 10.1016/j.immuni.2011.03.026

De Andrea, M., Ravera, R., Gioia, D., Gariglio, M., and Landolfo, S. (2002). The interferon system: an overview. *European Journal of Paediatric Neurology*, *6*, A41–A46. doi: 10.1053/ejpn.2002.0573

Arimoto, K., Konishi, H., and Shimotohno, K. (2008). UbcH8 regulates ubiquitin and ISG15 conjugation to RIG-I. *Molecular immunology*, *45*, 1078–1084. doi: 10.1016/j.molimm.2007.07.021

Arimoto, K., Takahashi, H., Hishiki, T., Konishi, H., Fujita, T., and Shimotohno, K. (2007). Negative regulation of the RIG-I signaling by the ubiquitin ligase RNF125. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(18), 7500–5. doi: 10.1073/pnas.0611551104

Arnold, M. M., and Patton, J. T. (2011). Diversity of interferon antagonist activities mediated by NSP1 proteins of different rotavirus strains. *Journal of virology*, *85*(5), 1970–9. doi: 10.1128/JVI.01801-10

Au, W. C. (1998). Characterization of the Interferon Regulatory Factor-7 and Its Potential Role in the Transcription Activation of Interferon A Genes. *Journal of Biological Chemistry*, *273*(44), 29210–29217. doi: 10.1074/jbc.273.44.29210

Au, W. C., Moore, P. a, Lowther, W., Juang, Y. T., and Pitha, P. M. (1995). Identification of a member of the interferon regulatory factor family that binds to the interferon-stimulated response element and activates expression of interferon-induced genes. *Proceedings of the National Academy of Sciences of the United States of America*, *92*(25), 11657–61.

Backert, S., Feller, S. M., and Wessler, S. (2008). Emerging roles of Abl family tyrosine kinases in microbial pathogenesis. *Trends in biochemical sciences*, *33*(2), 80–90. doi: 10.1016/j.tibs.2007.10.006

Baker, M., Frazier, A., Gulbis, J., and Ryan, M. (2007). Mitochondrial protein-import machinery: correlating structure with function. *Trends in cell biology*, *17*(9). doi: 10.1016/j.tcb.2007.07.010

Balachandran, S., Thomas, E., and Barber, G. N. (2004). A FADD-dependent innate immune mechanism in mammalian cells. *Nature*, *432*(7015), 401–5. doi: 10.1038/nature03124

Baltimore, D. (1971). Expression of animal virus genomes. *Bacteriological reviews*, *35*(3), 235–41.

Bannister, A. J., and Kouzarides, T. (2011). Regulation of chromatin by histone modifications. *Cell research*, *21*(3), 381–95. Nature Publishing Group. doi: 10.1038/cr.2011.22

Barnes, B. J., Moore, P. a, and Pitha, P. M. (2001). Virus-specific activation of a novel interferon regulatory factor, IRF-5, results in the induction of distinct interferon alpha genes. *The Journal of biological chemistry*, *276*(26), 23382–90. doi: 10.1074/jbc.M101216200

Belgnaoui, S. M., Paz, S., and Hiscott, J. (2011). Orchestrating the interferon antiviral response through the mitochondrial antiviral signaling (MAVS) adapter. *Current opinion in immunology*, *23*(5), 564–72. Elsevier Ltd. doi: 10.1016/j.coi.2011.08.001

Bellingham, J., Gregory-Evans, K., and Gregory-Evans, C. Y. (1998). Mapping of human interferon regulatory factor 3 (IRF3) to chromosome 19q13.3-13.4 by an intragenic polymorphic marker. *Annals of human genetics*, *62*(Pt 3), 231–4. doi: 10.1046/j.1469-1809.1998.6230231.x

Benjamin, R., Sharma, S., Zou, W., Sun, Q., Grandvaux, N., Julkunen, I., Hemmi, H., et al. (2004). Activation of TBK1 and IKKepsilon Kinases by Vesicular Stomatitis Virus Infection and the Role of Viral Ribonucleoprotein in the Development of Interferon Antiviral Immunity. *J. Virol.*, *78*(19), 10636–10649. doi: 10.1128/JVI.78.19.10636

Berkowitz, B., Huang, D.-B., Chen-Park, F. E., Sigler, P. B., and Ghosh, G. (2002). The x-ray crystal structure of the NF-kappa B p50.p65 heterodimer bound to the interferon beta -kappa B site. *The Journal of biological chemistry*, *277*(27), 24694–700. doi: 10.1074/jbc.M200006200

Bhanja Chowdhury, J., Shrivastava, S., Steele, R., Di Bisceglie, A. M., Ray, R., and Ray, R. B. (2012). Hepatitis C virus infection modulates expression of interferon stimulatory gene IFITM1 by upregulating miR-130A. *Journal of virology*, *86*(18), 10221–5. doi: 10.1128/JVI.00882-12

Van der Bliek, A. M., Shen, Q., and Kawajiri, S. (2013). Mechanisms of mitochondrial fission and fusion. *Cold Spring Harbor perspectives in biology*, *5*(6), 1–16. doi: 10.1101/cshperspect.a011072

Bode, J. G., Ludwig, S., Ehrhardt, C., Albrecht, U., Erhardt, A., Schaper, F., Heinrich, P. C., et al. (2003). IFN-alpha antagonistic activity of HCV core protein involves induction of suppressor of cytokine signaling-3. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, *17*(3), 488–90. doi: 10.1096/fj.02-0664fje

Boonyaratanakornkit, J., Bartlett, E., Schomacker, H., Surman, S., Akira, S., Bae, Y.-S., Collins, P. L., et al. (2011). The C proteins of human parainfluenza virus type 1

limit double-stranded RNA accumulation that would otherwise trigger activation of MDA5 and protein kinase R. *Journal of virology*, *7*(4), e28382. doi: 10.1128/JVI.01297-10

Borrow, P., Martínez-Sobrido, L., and de la Torre, J. C. (2010). Inhibition of the type I interferon antiviral response during arenavirus infection. *Viruses*, *2*(11), 2443–80. doi: 10.3390/v2112443

Bozym, R. a, Delorme-Axford, E., Harris, K., Morosky, S., Ikizler, M., Dermody, T. S., Sarkar, S. N., et al. (2012). Focal adhesion kinase is a component of antiviral RIG-I-like receptor signaling. *Cell host & microbe*, *11*(2), 153–66. Elsevier Inc. doi: 10.1016/j.chom.2012.01.008

Bürckstümmer, T., Baumann, C., Blüml, S., Dixit, E., Dürnberger, G., Jahn, H., Planyavsky, M., et al. (2009). An orthogonal proteomic-genomic screen identifies AIM2 as a cytoplasmic DNA sensor for the inflammasome. *Nature immunology*, *10*(3), 266–72. doi: 10.1038/ni.1702

Caillaud, A., and Hovanessian, A. (2005). Regulatory serine residues mediate phosphorylation-dependent and phosphorylation-independent activation of interferon regulatory factor 7. *Journal of Biological …*, *280*(18), 17671–17677.

Camões, F., Bonekamp, N. a, Delille, H. K., and Schrader, M. (2009). Organelle dynamics and dysfunction: A closer link between peroxisomes and mitochondria. *Journal of inherited metabolic disease*, *32*(2), 163–80. doi: 10.1007/s10545-008-1018-3

Castanier, C., and Arnoult, D. (2011). Mitochondrial localization of viral proteins as a means to subvert host defense. *Biochimica et biophysica acta*, *1813*(4), 575–83. Elsevier B.V. doi: 10.1016/j.bbamcr.2010.08.009

Castanier, C., Garcin, D., Vazquez, A., and Arnoult, D. (2010). Mitochondrial dynamics regulate the RIG-I-like receptor antiviral pathway. *EMBO reports*, *11*(2), 133–8. doi: 10.1038/embor.2009.258

Castanier, C., Zemirli, N., Portier, A., Garcin, D., Bidère, N., Vazquez, A., and Arnoult, D. (2012). MAVS ubiquitination by the E3 ligase TRIM25 and degradation by the proteasome is involved in type I interferon production after activation of the antiviral RIG-I-like receptors. *BMC biology*, *10*(1), 44. BioMed Central Ltd. doi: 10.1186/1741-7007-10-44

Childs, K., Randall, R., and Goodbourn, S. (2012). Paramyxovirus V proteins interact with the RNA Helicase LGP2 to inhibit RIG-I-dependent interferon induction. *Journal of virology*, *86*(7), 3411–21. doi: 10.1128/JVI.06405-11

Chinenov, Y., and Kerppola, T. (2001). Close encounters of many kinds: Fos-Jun interactions that mediate transcription regulatory specificity. *Oncogene*, 2438–2452.

Choudhary, C., Kumar, C., Gnad, F., Nielsen, M. L., Rehman, M., Walther, T. C., Olsen, J. V, et al. (2009). Lysine acetylation targets protein complexes and co-regulates major cellular functions. *Science*, *325*(5942), 834–40. doi: 10.1126/science.1175371

Cooper, G. (2000). *The Cell: A Molecular Approach* (2nd ed.). Sunderland (MA): Sinauer Associates.

Cui, S., Eisenächer, K., Kirchhofer, A., Brzózka, K., Lammens, A., Lammens, K., Fujita, T., et al. (2008). The C-terminal regulatory domain is the RNA 5'-triphosphate sensor of RIG-I. *Molecular cell*, *29*(2), 169–79. doi: 10.1016/j.molcel.2007.10.032

Delille, H. K., Alves, R., and Schrader, M. (2009). Biogenesis of peroxisomes and mitochondria: linked by division. *Histochemistry and cell biology*, *131*(4), 441–6. doi: 10.1007/s00418-009-0561-9

Devasthanam, A. S. (2014). Mechanisms underlying the inhibition of interferon signaling by viruses. *Virulence*, *5*(2), 270–277. doi: 10.4161/viru.27902

Diao, F., Li, S., Tian, Y., Zhang, M., Xu, L.-G., Zhang, Y., Wang, R.-P., et al. (2007). Negative regulation of MDA5- but not RIG-I-mediated innate antiviral signaling by the dihydroxyacetone kinase. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(28), 11706–11. doi: 10.1073/pnas.0700544104

Dixit, E., Boulant, S., Zhang, Y., Lee, A. S. Y., Odendall, C., Shum, B., Hacohen, N., et al. (2010). Peroxisomes are signaling platforms for antiviral innate immunity. *Cell*, *141*(4), 668–81. doi: 10.1016/j.cell.2010.04.018

Dixit, E., and Kagan, J. C. (2013). *Intracellular pathogen detection by RIG-I-like receptors. Advances in immunology* (1st ed., Vol. 117, pp. 99–125). Elsevier Inc. doi: 10.1016/B978-0-12-410524-9.00004-9

Ernster, L., and Schatz, G. (1981). Mitochondria: a historical review. *The Journal of cell biology*, *91*(3), 227–255.

Escalante, C. R., Yie, J., Thanos, D., and Aggarwal, a K. (1998). Structure of IRF-1 with bound DNA reveals determinants of interferon regulation. *Nature*, *391*(6662), 103–6. doi: 10.1038/34224

ExPASy. (2014). ViralZone. Retrieved March 17, 2014, from http://viralzone.expasy.org/

Falvo, J. V, Thanos, D., and Maniatis, T. (1995). Reversal of intrinsic DNA bends in the IFN beta gene enhancer by transcription factors and the architectural protein HMG I(Y). *Cell*, *83*(7), 1101–11.

Fan, T.-J., Han, L.-H., Cong, R.-S., and Liang, J. (2005). Caspase Family Proteases and Apoptosis. *Acta Biochimica et Biophysica Sinica*, *37*(11), 719–727. doi: 10.1111/j.1745-7270.2005.00108.x

Fensterl, V., and Sen, G. C. (2009). Interferons and viral infections. *BioFactors (Oxford, England)*, *35*(1), 14–20. doi: 10.1002/biof.6

Fernandes-Alnemri, T., Yu, J.-W., Datta, P., Wu, J., and Alnemri, E. S. (2009). AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA. *Nature*, *458*(7237), 509–13. Nature Publishing Group. doi: 10.1038/nature07710

Fonseca, G. J., Thillainadesan, G., Yousef, a F., Ablack, J. N., Mossman, K. L., Torchia, J., and Mymryk, J. S. (2012). Adenovirus evasion of interferon-mediated innate immunity by direct antagonism of a cellular histone posttranslational modification. *Cell host & microbe*, *11*(6), 597–606. Elsevier Inc. doi: 10.1016/j.chom.2012.05.005

Ford, E., and Thanos, D. (2010). The transcriptional code of human IFN-beta gene expression. *Biochimica et biophysica acta*, *1799*(3-4), 328–36. Elsevier B.V. doi: 10.1016/j.bbagrm.2010.01.010

Frias, A. H., Jones, R. M., Fifadara, N. H., Vijay-Kumar, M., and Gewirtz, A. T. (2012). Rotavirus-induced IFN-β promotes anti-viral signaling and apoptosis that modulate viral replication in intestinal epithelial cells. *Innate immunity*, *18*(2), 294–306. doi: 10.1177/1753425911401930

Friedman, C. S., O'Donnell, M. A., Legarda-Addison, D., Ng, A., Cárdenas, W. B., Yount, J. S., Moran, T. M., et al. (2008). The tumour suppressor CYLD is a negative regulator of RIG-I-mediated antiviral response. *EMBO reports*, *9*(9), 930–6. doi: 10.1038/embor.2008.136

Fujita, T., Kimura, Y., and Miyamoto, M. (1989). Induction of endogenous IFN-α and IFN-β genes by a regulatory transcription factor, IRF-1. *Nature*.

Gack, M. U., Nistal-Villán, E., Inn, K.-S., García-Sastre, A., and Jung, J. U. (2010). Phosphorylation-mediated negative regulation of RIG-I antiviral activity. *Journal of virology*, *84*(7), 3220–9. doi: 10.1128/JVI.02241-09

Gack, M. U., Shin, Y. C., Joo, C.-H., Urano, T., Liang, C., Sun, L., Takeuchi, O., et al. (2007). TRIM25 RING-finger E3 ubiquitin ligase is essential for RIG-I-mediated antiviral activity. *Nature*, *446*(7138), 916–920. doi: 10.1038/nature05732

Garcin, D., and Marq, J. (2003). The amino-terminal extensions of the longer Sendai virus C proteins modulate pY701-Stat1 and bulk Stat1 levels independently of interferon signaling. *Journal of virology*. doi: 10.1128/JVI.77.4.2321

Ghosh, S., and Hayden, M. S. (2008). New regulators of NF-kappaB in inflammation. *Nature reviews. Immunology*, *8*(11), 837–48. doi: 10.1038/nri2423

Gilmore, T. (2014). NF-kB Transcription Factors. Retrieved January 28, 2014, from http://www.bu.edu/nf-kb/

Gitlin, L., Barchet, W., Gilfillan, S., Cella, M., Beutler, B., Flavell, R. a, Diamond, M. S., et al. (2006). Essential role of mda-5 in type I IFN responses to polyriboinosinic:polyribocytidylic acid and encephalomyocarditis picornavirus. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(22), 8459–64. doi: 10.1073/pnas.0603082103

Gori-Savellini, G., Valentini, M., and Cusi, M. G. (2013). Toscana virus NSs protein inhibits the induction of type I interferon by interacting with RIG-I. *Journal of virology*, *87*(12), 6660–7. doi: 10.1128/JVI.03129-12

Gotoh, B., Takeuchi, K., Komatsu, T., and Yokoo, J. (2003). The STAT2 activation process is a crucial target of Sendai virus C protein for the blockade of alpha interferon signaling. *Journal of virology*. doi: 10.1128/JVI.77.6.3360

Guo, B., and Cheng, G. (2007). Modulation of the interferon antiviral response by the TBK1/IKKi adaptor protein TANK. *The Journal of biological chemistry*, *282*(16), 11817–26. doi: 10.1074/jbc.M700017200

Häcker, H., Redecke, V., Blagoev, B., Kratchmarova, I., Hsu, L.-C., Wang, G. G., Kamps, M. P., et al. (2006). Specificity in Toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6. *Nature*, *439*(7073), 204–7. doi: 10.1038/nature04369

Halfmann, P., Neumann, G., and Kawaoka, Y. (2011). The Ebolavirus VP24 protein blocks phosphorylation of p38 mitogen-activated protein kinase. *The Journal of infectious diseases*, *204 Suppl*(Suppl 3), S953–6. doi: 10.1093/infdis/jir325

Hall, J. E. (2010). *Guyton and Hall Textbook of Medical Physiology*. *Physiology* (12th ed., p. 1120). Saunders.

Haller, O., Kochs, G., and Weber, F. (2006). The interferon response circuit: induction and suppression by pathogenic viruses. *Virology*, *344*(1), 119–30. doi: 10.1016/j.virol.2005.09.024

Hastie, K. M., Kimberlin, C. R., Zandonatti, M. A., MacRae, I. J., and Saphire, E. O. (2011). Structure of the Lassa virus nucleoprotein reveals a dsRNA-specific 3’ to 5' exonuclease activity essential for immune suppression. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(6), 2396–401. doi: 10.1073/pnas.1016404108

Hayakawa, S., Shiratori, S., Yamato, H., Kameyama, T., Kitatsuji, C., Kashigi, F., Goto, S., et al. (2011). ZAPS is a potent stimulator of signalling mediated by the RNA helicase RIG-I during antiviral responses. *Nature Immunology*, (12), 37–44.

Hayden, M. S., and Ghosh, S. (2008). Shared principles in NF-kappaB signaling. *Cell*, *132*(3), 344–62. doi: 10.1016/j.cell.2008.01.020

Hemler, M. E. (2005). Tetraspanin functions and associated microdomains. *Nature reviews. Molecular cell biology*, *6*(10), 801–11. doi: 10.1038/nrm1736

Hemmi, H., Takeuchi, O., Sato, S., Yamamoto, M., Kaisho, T., Sanjo, H., Kawai, T., et al. (2004). The roles of two IkappaB kinase-related kinases in lipopolysaccharide and double stranded RNA signaling and viral infection. *The Journal of experimental medicine*, *199*(12), 1641–50. doi: 10.1084/jem.20040520

Hiscott, J. (2007a). Convergence of the NF-kappaB and IRF pathways in the regulation of the innate antiviral response. *Cytokine & growth factor reviews*, *18*(5-6), 483–90. doi: 10.1016/j.cytogfr.2007.06.002

Hiscott, J. (2007b). Triggering the innate antiviral response through IRF-3 activation. *The Journal of biological chemistry*, *282*(21), 15325–9. doi: 10.1074/jbc.R700002200

Honda, K., Takaoka, A., and Taniguchi, T. (2006). Type I interferon gene induction by the interferon regulatory factor family of transcription factors. *Immunity*, *25*(3), 349–60. doi: 10.1016/j.immuni.2006.08.009

Honda, K., Yanai, H., Takaoka, A., and Taniguchi, T. (2005). Regulation of the type I IFN induction: a current view. *International immunology*, *17*(11), 1367–78. doi: 10.1093/intimm/dxh318

Hong, S., Mehta, K. P., and Laimins, L. a. (2011). Suppression of STAT-1 expression by human papillomaviruses is necessary for differentiation-dependent genome amplification and plasmid maintenance. *Journal of virology*, *85*(18), 9486–94. doi: 10.1128/JVI.05007-11

Horner, S. M., Liu, H. M., Park, H. S., Briley, J., and Gale, M. (2011). Mitochondrial-associated endoplasmic reticulum membranes (MAM) form innate immune synapses and are targeted by hepatitis C virus. *Proceedings of the National*

Academy of Sciences of the United States of America, *108*(35), 14590–5. doi: 10.1073/pnas.1110133108

Hornung, V., Ablasser, A., Charrel-Dennis, M., Bauernfeind, F., Horvath, G., Caffrey, D. R., Latz, E., et al. (2009). AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. *Nature*, *458*(7237), 514–8. Nature Publishing Group. doi: 10.1038/nature07725

Hornung, V., Ellegast, J., Kim, S., Brzózka, K., Jung, A., Kato, H., Poeck, H., et al. (2006). 5'-Triphosphate RNA is the ligand for RIG-I. *Science*, *314*(5801), 994–7. doi: 10.1126/science.1132505

Hou, F., Sun, L., Zheng, H., Skaug, B., Jiang, Q.-X., and Chen, Z. J. (2011). MAVS forms functional prion-like aggregates to activate and propagate antiviral innate immune response. *Cell*, *146*(3), 448–61. Elsevier Inc. doi: 10.1016/j.cell.2011.06.041

Hoving, J. C., Wilson, G. J., and Brown, G. D. (2014). Signalling C-type lectin receptors, microbial recognition and immunity. *Cellular microbiology*, *16*(2), 185–94. doi: 10.1111/cmi.12249

Huang, J., Liu, T., Xu, L.-G., Chen, D., Zhai, Z., and Shu, H.-B. (2005). SIKE is an IKK epsilon/TBK1-associated suppressor of TLR3- and virus-triggered IRF-3 activation pathways. *The EMBO journal*, *24*(23), 4018–28. doi: 10.1038/sj.emboj.7600863

Inn, K.-S., Lee, S.-H., Rathbun, J. Y., Wong, L.-Y., Toth, Z., Machida, K., Ou, J.-H. J., et al. (2011). Inhibition of RIG-I-mediated signaling by Kaposi's sarcoma-associated herpesvirus-encoded deubiquitinase ORF64. *Journal of virology*, *85*(20), 10899–904. doi: 10.1128/JVI.00690-11

International Committee on Taxonomy of Viruses. (2013). Virus Taxonomy: 2013 Release. *July 2013*. Retrieved March 17, 2014, from http://ictvonline.org/virusTaxonomy.asp

Ishikawa, H., and Barber, G. N. (2008). STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. *Nature*, *455*(7213), 674–8. doi: 10.1038/nature07317

Island, M., and Mesplede, T. (2002). Repression by homeoprotein pitx1 of virus-induced interferon a promoters is mediated by physical interaction and trans repression of IRF3 and IRF7. *Molecular and cellular biology*, *22*(20), 7120–7133. doi: 10.1128/MCB.22.20.7120

Islinger, M., and Schrader, M. (2011). Peroxisomes. *Current biology : CB*, *21*(19), R800–1. doi: 10.1016/j.cub.2011.07.024

Jacobs, J. L., and Coyne, C. B. (2013). Mechanisms of MAVS Regulation at the Mitochondrial Membrane. *Journal of molecular biology*. Elsevier B.V. doi: 10.1016/j.jmb.2013.10.007

Jeffrey, K. L., Camps, M., Rommel, C., and Mackay, C. R. (2007). Targeting dual-specificity phosphatases: manipulating MAP kinase signalling and immune responses. *Nature reviews. Drug discovery*, *6*(5), 391–403. doi: 10.1038/nrd2289

Jia, Y., Song, T., Wei, C., Ni, C., Zheng, Z., Xu, Q., Ma, H., et al. (2009). Negative regulation of MAVS-mediated innate immune response by PSMA7. *Journal of*

immunology (Baltimore, Md. : 1950), *183*(7), 4241–8. doi: 10.4049/jimmunol.0901646

Jiang, H., and Fisher, P. B. (1993). Use of a sensitive and efficient subtraction hybridization protocol for the identification of genes differentially regulated during the induction of differentiation in human melanoma cells. *Mol. Cell. Different.*, *1*(3), 285–299.

Jiang, X., Kinch, L. N., Brautigam, C. a, Chen, X., Du, F., Grishin, N. V, and Chen, Z. J. (2012). Ubiquitin-induced oligomerization of the RNA sensors RIG-I and MDA5 activates antiviral innate immune response. *Immunity*, *36*(6), 959–73. Elsevier Inc. doi: 10.1016/j.immuni.2012.03.022

Jin, L., Hill, K. K., Filak, H., Mogan, J., Knowles, H., Zhang, B., Perraud, A.-L., et al. (2011). MPYS is required for IFN response factor 3 activation and type I IFN production in the response of cultured phagocytes to bacterial second messengers cyclic-di-AMP and cyclic-di-GMP. *Journal of immunology (Baltimore, Md. : 1950)*, *187*(5), 2595–601. doi: 10.4049/jimmunol.1100088

Jin, L., Waterman, P. M., Jonscher, K. R., Short, C. M., Reisdorph, N. a, and Cambier, J. C. (2008). MPYS, a novel membrane tetraspanner, is associated with major histocompatibility complex class II and mediates transduction of apoptotic signals. *Molecular and cellular biology*, *28*(16), 5014–26. doi: 10.1128/MCB.00640-08

Johnsen, I. B., Nguyen, T. T., Bergstroem, B., Fitzgerald, K. a, and Anthonsen, M. W. (2009). The tyrosine kinase c-Src enhances RIG-I (retinoic acid-inducible gene I)-elicited antiviral signaling. *The Journal of biological chemistry*, *284*(28), 19122–31. doi: 10.1074/jbc.M808233200

Jounai, N., Takeshita, F., Kobiyama, K., Sawano, A., Miyawaki, A., Xin, K.-Q., Ishii, K. J., et al. (2007). The Atg5 Atg12 conjugate associates with innate antiviral immune responses. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(35), 14050–5. doi: 10.1073/pnas.0704014104

Kadeppagari, R.-K., Sanchez, R. L., and Foster, T. P. (2012). HSV-2 inhibits type-I interferon signaling via multiple complementary and compensatory STAT2-associated mechanisms. *Virus research*, *167*(2), 273–84. Elsevier B.V. doi: 10.1016/j.virusres.2012.05.010

Kanneganti, T.-D. (2010). Central roles of NLRs and inflammasomes in viral infection. *Nature reviews Immunology*, *10*(10), 688–98. Nature Publishing Group. doi: 10.1038/nri2851

Van Kasteren, P. B., Beugeling, C., Ninaber, D. K., Frias-Staheli, N., van Boheemen, S., García-Sastre, A., Snijder, E. J., et al. (2012). Arterivirus and nairovirus ovarian tumor domain-containing Deubiquitinases target activated RIG-I to control innate immune signaling. *Journal of virology*, *86*(2), 773–85. doi: 10.1128/JVI.06277-11

Kato, H., Takeuchi, O., Mikamo-Satoh, E., Hirai, R., Kawai, T., Matsushita, K., Hiiragi, A., et al. (2008). Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5. *The Journal of experimental medicine*, *205*(7), 1601–10. doi: 10.1084/jem.20080091

Kawai, T., and Akira, S. (2011). Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity*, *34*(5), 637–50. Elsevier Inc. doi: 10.1016/j.immuni.2011.05.006

Kawai, T., Takahashi, K., Sato, S., Coban, C., Kumar, H., Kato, H., Ishii, K. J., et al. (2005). IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. *Nature immunology*, *6*(10), 981–8. doi: 10.1038/ni1243

Kayagaki, N., Phung, Q., Chan, S., Chaudhari, R., Quan, C., O'Rourke, K. M., Eby, M., et al. (2007). DUBA: a deubiquitinase that regulates type I interferon production. *Science*, *318*(5856), 1628–32. doi: 10.1126/science.1145918

Kim, M., and Yoo, J. (2008). Active caspase-1-mediated secretion of retinoic acid inducible gene-I. *The Journal of Immunology*, (181), 7324–73.

Kim, T. K., Kim, T. H., and Maniatis, T. (1998). Efficient recruitment of TFIIB and CBP-RNA polymerase II holoenzyme by an interferon-beta enhanceosome in vitro. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(21), 12191–6.

Kindt, T. J., Osborne, B. A., and Goldsby, R. A. (2007). *Kuby Immunology* (6th ed.). W. H. Freeman & Company.

Kishore, N., Huynh, Q. K., Mathialagan, S., Hall, T., Rouw, S., Creely, D., Lange, G., et al. (2002). IKK-i and TBK-1 are enzymatically distinct from the homologous enzyme IKK-2: comparative analysis of recombinant human IKK-i, TBK-1, and IKK-2. *The Journal of biological chemistry*, *277*(16), 13840–7. doi: 10.1074/jbc.M110474200

Koleske, A. J. (2006). *Abl Family Kinases in Development and Disease*. Molecular Biology Intelligence Unit. New York, NY: Springer New York. doi: 10.1007/978-0-387-68744-5

Komuro, A., Bamming, D., and Horvath, C. M. (2008). Negative regulation of cytoplasmic RNA-mediated antiviral signaling. *Cytokine*, *43*(3), 350–8. doi: 10.1016/j.cyto.2008.07.011

Komuro, A., and Horvath, C. M. (2006). RNA- and virus-independent inhibition of antiviral signaling by RNA helicase LGP2. *Journal of virology*, *80*(24), 12332–42. doi: 10.1128/JVI.01325-06

Koshiba, T., Yasukawa, K., Yanagi, Y., and Kawabata, S. (2011). Mitochondrial membrane potential is required for MAVS-mediated antiviral signaling. *Science signaling*, *4*(158), ra7. doi: 10.1126/scisignal.2001147

Kouzarides, T. (2007). Chromatin modifications and their function. *Cell*, *128*(4), 693–705. doi: 10.1016/j.cell.2007.02.005

Kowalinski, E., Lunardi, T., McCarthy, A. a, Louber, J., Brunel, J., Grigorov, B., Gerlier, D., et al. (2011). Structural basis for the activation of innate immune pattern-recognition receptor RIG-I by viral RNA. *Cell*, *147*(2), 423–35. Elsevier Inc. doi: 10.1016/j.cell.2011.09.039

Koyama, S., Ishii, K. J., Coban, C., and Akira, S. (2008). Innate immune response to viral infection. *Cytokine*, *43*(3), 336–41. doi: 10.1016/j.cyto.2008.07.009

Ku, C.-C., Che, X.-B., Reichelt, M., Rajamani, J., Schaap-Nutt, A., Huang, K.-J., Sommer, M. H., et al. (2011). Herpes simplex virus-1 induces expression of a novel MxA isoform that enhances viral replication. *Immunology and cell biology*, *89*(2), 173–82. Nature Publishing Group. doi: 10.1038/icb.2010.83

Kubota, T., Matsuoka, M., Chang, T.-H., Tailor, P., Sasaki, T., Tashiro, M., Kato, A., et al. (2008). Virus infection triggers SUMOylation of IRF3 and IRF7, leading to the negative regulation of type I interferon gene expression. *The Journal of biological chemistry*, *283*(37), 25660–70. doi: 10.1074/jbc.M804479200

Kumar, M., Jung, S. Y., Hodgson, A. J., Madden, C. R., Qin, J., and Slagle, B. L. (2011). Hepatitis B virus regulatory HBx protein binds to adaptor protein IPS-1 and inhibits the activation of beta interferon. *Journal of virology*, *85*(2), 987–95. doi: 10.1128/JVI.01825-10

Leung, L. W., Park, M.-S., Martinez, O., Valmas, C., López, C. B., and Basler, C. F. (2011). Ebolavirus VP35 suppresses IFN production from conventional but not plasmacytoid dendritic cells. *Immunology and cell biology*, *89*(7), 792–802. doi: 10.1038/icb.2010.169

Levy, D. E., and Marié, I. J. (2005). HOW VIRUSES ELICIT INTERFERON - Triggering the innate immune response to viral infection. *Modulation of Host Gene Expression and Innate Immunity by Viruses* (pp. 19–34). Springer.

Li, S., Zheng, H., Mao, A.-P., Zhong, B., Li, Y., Liu, Y., Gao, Y., et al. (2010). Regulation of virus-triggered signaling by OTUB1- and OTUB2-mediated deubiquitination of TRAF3 and TRAF6. *The Journal of biological chemistry*, *285*(7), 4291–7. doi: 10.1074/jbc.M109.074971

Li, X.-D., Sun, L., Seth, R. B., Pineda, G., and Chen, Z. J. (2005). Hepatitis C virus protease NS3/4A cleaves mitochondrial antiviral signaling protein off the mitochondria to evade innate immunity. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(49), 17717–22. doi: 10.1073/pnas.0508531102

Liang, D., Gao, Y., Lin, X., He, Z., Zhao, Q., Deng, Q., and Lan, K. (2011). A human herpesvirus miRNA attenuates interferon signaling and contributes to maintenance of viral latency by targeting IKKε. *Cell research*, *21*(5), 793–806. Nature Publishing Group. doi: 10.1038/cr.2011.5

Liang, Q., Fu, B., Wu, F., Li, X., Yuan, Y., and Zhu, F. (2012). ORF45 of Kaposi's sarcoma-associated herpesvirus inhibits phosphorylation of interferon regulatory factor 7 by IKKε and TBK1 as an alternative substrate. *Journal of virology*, *86*(18), 10162–72. doi: 10.1128/JVI.05224-11

Lifland, A. W., Jung, J., Alonas, E., Zurla, C., Crowe, J. E., and Santangelo, P. J. (2012). Human respiratory syncytial virus nucleoprotein and inclusion bodies antagonize the innate immune response mediated by MDA5 and MAVS. *Journal of virology*, *86*(15), 8245–58. doi: 10.1128/JVI.00215-12

Lin, R., Heylbroeck, C., Pitha, P. M., and Hiscott, J. (1998). Virus-dependent phosphorylation of the IRF-3 transcription factor regulates nuclear translocation, transactivation potential, and proteasome-mediated degradation. *Molecular and cellular biology*, *18*(5), 2986–96.

Lin, R., Mamane, Y., and Hiscott, J. (1999). Structural and functional analysis of interferon regulatory factor 3: localization of the transactivation and autoinhibitory domains. *Molecular and cellular biology*, *19*(4), 2465–74.

Lin, R., Mamane, Y., and Hiscott, J. (2000). Multiple regulatory domains control IRF-7 activity in response to virus infection. *The Journal of biological chemistry*, *275*(44), 34320–7. doi: 10.1074/jbc.M002814200

Lin, R., Yang, L., Nakhaei, P., Sun, Q., Sharif-Askari, E., Julkunen, I., and Hiscott, J. (2006). Negative regulation of the retinoic acid-inducible gene I-induced antiviral state by the ubiquitin-editing protein A20. *The Journal of biological chemistry*, *281*(4), 2095–103. doi: 10.1074/jbc.M510326200

Ling, A., Soares, F., Croitoru, D. O., Tattoli, I., Carneiro, L. a M., Boniotto, M., Benko, S., et al. (2012). Post-transcriptional inhibition of luciferase reporter assays by the Nod-like receptor proteins NLRX1 and NLRC3. *The Journal of biological chemistry*, *287*(34), 28705–16. doi: 10.1074/jbc.M111.333146

Liu, S., Chen, J., Cai, X., Wu, J., Chen, X., Wu, Y.-T., Sun, L., et al. (2013). MAVS recruits multiple ubiquitin E3 ligases to activate antiviral signaling cascades. *eLife*, *2*, e00785. doi: 10.7554/eLife.00785

Liu, X.-Y., Chen, W., Wei, B., Shan, Y.-F., and Wang, C. (2011). IFN-induced TPR protein IFIT3 potentiates antiviral signaling by bridging MAVS and TBK1. *Journal of immunology (Baltimore, Md. : 1950)*, *187*(5), 2559–68. doi: 10.4049/jimmunol.1100963

Liu, X.-Y., Wei, B., Shi, H.-X., Shan, Y.-F., and Wang, C. (2010). Tom70 mediates activation of interferon regulatory factor 3 on mitochondria. *Cell research*, *20*(9), 994–1011. Nature Publishing Group. doi: 10.1038/cr.2010.103

Lodish, H. F., Berk, A., Zipursky, S. L., Matsudaira, P., Baltimore, D., and James, D. (2008). *Molecular Cell Biology*. *Book* (Vol. 5). doi: 10.1016/S1470-8175(01)00023-6

Lomvardas, S., and Thanos, D. (2001). Nucleosome sliding via TBP DNA binding in vivo. *Cell*, *106*(6), 685–96.

Loo, Y.-M., and Gale, M. (2011). Immune signaling by RIG-I-like receptors. *Immunity*, *34*(5), 680–92. Elsevier Inc. doi: 10.1016/j.immuni.2011.05.003

Luo, D., Ding, S. C., Vela, A., Kohlway, A., Lindenbach, B. D., and Pyle, A. M. (2011). Structural insights into RNA recognition by RIG-I. *Cell*, *147*, 409–422. doi: 10.1016/j.cell.2011.09.023

Ma, Y., Jin, H., Valyi-Nagy, T., Cao, Y., Yan, Z., and He, B. (2012). Inhibition of TANK binding kinase 1 by herpes simplex virus 1 facilitates productive infection. *Journal of virology*, *86*(4), 2188–96. doi: 10.1128/JVI.05376-11

Makeyev, A., and Liebhaber, S. (2002). The poly (C)-binding proteins: a multiplicity of functions and a search for mechanisms. *Rna*, (8), 265–278. doi: 10+1017+S1355838202024627

Mamane, Y., Heylbroeck, C., Génin, P., Algarté, M., Servant, M. J., LePage, C., DeLuca, C., et al. (1999). Interferon regulatory factors: the next generation. *Gene*, *237*(1), 1–14.

Marazzi, I., Ho, J., Kim, J., and Manicassamy, B. (2012). Suppression of the antiviral response by an influenza histone mimic. *Nature*, *483*(7390), 428–33. doi: 10.1038/nature10892

Marié, I., Durbin, J. E., and Levy, D. E. (1998). Differential viral induction of distinct interferon-alpha genes by positive feedback through interferon regulatory factor-7. *The EMBO journal*, *17*(22), 6660–9. doi: 10.1093/emboj/17.22.6660

Matsui, K., Kumagai, Y., Kato, H., Sato, S., Kawagoe, T., Uematsu, S., Takeuchi, O., et al. (2006). Cutting edge: Role of TANK-binding kinase 1 and inducible IkappaB kinase in IFN responses against viruses in innate immune cells. *Journal of immunology (Baltimore, Md. : 1950)*, *177*(9), 5785–9.

McWhirter, S. M., Fitzgerald, K. a, Rosains, J., Rowe, D. C., Golenbock, D. T., and Maniatis, T. (2004). IFN-regulatory factor 3-dependent gene expression is defective in Tbk1-deficient mouse embryonic fibroblasts. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(1), 233–8. doi: 10.1073/pnas.2237236100

Meager, A. (2006). *The interferons: characterization and application*.

Merika, M., Williams, a J., Chen, G., Collins, T., and Thanos, D. (1998). Recruitment of CBP/p300 by the IFN beta enhanceosome is required for synergistic activation of transcription. *Molecular cell*, *1*(2), 277–87.

Meylan, E., Curran, J., Hofmann, K., Moradpour, D., Binder, M., Bartenschlager, R., and Tschopp, J. (2005). Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature*, *437*(7062), 1167–72. doi: 10.1038/nature04193

Michallet, M.-C., Meylan, E., Ermolaeva, M. a, Vazquez, J., Rebsamen, M., Curran, J., Poeck, H., et al. (2008). TRADD protein is an essential component of the RIG-like helicase antiviral pathway. *Immunity*, *28*(5), 651–61. doi: 10.1016/j.immuni.2008.03.013

Mikkelsen, S. S., Jensen, S. B., Chiliveru, S., Melchjorsen, J., Julkunen, I., Gaestel, M., Arthur, J. S. C., et al. (2009). RIG-I-mediated activation of p38 MAPK is essential for viral induction of interferon and activation of dendritic cells: dependence on TRAF2 and TAK1. *The Journal of biological chemistry*, *284*(16), 10774–82. doi: 10.1074/jbc.M807272200

Miorin, L., Albornoz, A., Baba, M. M., D'Agaro, P., and Marcello, A. (2012). Formation of membrane-defined compartments by tick-borne encephalitis virus contributes to the early delay in interferon signaling. *Virus research*, *163*(2), 660–6. Elsevier B.V. doi: 10.1016/j.virusres.2011.11.020

Moore, C. B., Bergstralh, D. T., Duncan, J. a, Lei, Y., Morrison, T. E., Zimmermann, A. G., Accavitti-Loper, M. a, et al. (2008). NLRX1 is a regulator of mitochondrial antiviral immunity. *Nature*, *451*(7178), 573–7. doi: 10.1038/nature06501

Mukherjee, A., Morosky, S. a, Delorme-Axford, E., Dybdahl-Sissoko, N., Oberste, M. S., Wang, T., and Coyne, C. B. (2011). The coxsackievirus B 3C protease cleaves MAVS and TRIF to attenuate host type I interferon and apoptotic signaling. *PLoS pathogens*, *7*(3), e1001311. doi: 10.1371/journal.ppat.1001311

Mukherjee, A., Morosky, S. a, Shen, L., Weber, C. R., Turner, J. R., Kim, K. S., Wang, T., et al. (2009). Retinoic acid-induced gene-1 (RIG-I) associates with the actin

cytoskeleton via caspase activation and recruitment domain-dependent interactions. *The Journal of biological chemistry*, *284*(10), 6486–94. doi: 10.1074/jbc.M807547200

Munshi, A., and Ramesh, R. (2013). Mitogen-Activated Protein Kinases and Their Role in Radiation Response. *Genes & cancer*, *4*(9-10), 401–408. doi: 10.1177/1947601913485414

Mutocheluh, M., Hindle, L., Aresté, C., Chanas, S. a, Butler, L. M., Lowry, K., Shah, K., et al. (2011). Kaposi's sarcoma-associated herpesvirus viral interferon regulatory factor-2 inhibits type 1 interferon signalling by targeting interferon-stimulated gene factor-3. *The Journal of general virology*, *92*(Pt 10), 2394–8. doi: 10.1099/vir.0.034322-0

Nakhaei, P., Mesplede, T., Solis, M., Sun, Q., Zhao, T., Yang, L., Chuang, T.-H., et al. (2009). The E3 ubiquitin ligase Triad3A negatively regulates the RIG-I/MAVS signaling pathway by targeting TRAF3 for degradation. *PLoS pathogens*, *5*(11), e1000650. doi: 10.1371/journal.ppat.1000650

Neznanov, N., Chumakov, K. M., Neznanova, L., Almasan, A., Banerjee, A. K., and Gudkov, A. V. (2005). Proteolytic cleavage of the p65-RelA subunit of NF-kappaB during poliovirus infection. *The Journal of biological chemistry*, *280*(25), 24153–8. doi: 10.1074/jbc.M502303200

Nguyen, H., Hiscott, J., and Pitha, P. M. (1997). The growing family of interferon regulatory factors. *Cytokine & growth factor reviews*, *8*(4), 293–312.

Odendall, C., Dixit, E., Stavru, F., Bierne, H., Franz, K. M., Durbin, A. F., Boulant, S., et al. (2014). Diverse intracellular pathogens activate type III interferon expression from peroxisomes. *Nature immunology*, (June). Nature Publishing Group. doi: 10.1038/ni.2915

Ohta, A., and Nishiyama, Y. (2011). Mitochondria and viruses. *Mitochondrion*, *11*(1), 1–12. Elsevier B.V. and Mitochondria Research Society. doi: 10.1016/j.mito.2010.08.006

Okabe, Y., Sano, T., and Nagata, S. (2009). Regulation of the innate immune response by threonine-phosphatase of Eyes absent. *Nature*, *460*(7254), 520–4. Nature Publishing Group. doi: 10.1038/nature08138

Onoguchi, K., Onomoto, K., Takamatsu, S., Jogi, M., Takemura, A., Morimoto, S., Julkunen, I., et al. (2010). Virus-infection or 5'ppp-RNA activates antiviral signal through redistribution of IPS-1 mediated by MFN1. *PLoS pathogens*, *6*(7), e1001012. doi: 10.1371/journal.ppat.1001012

Oshiumi, H., Matsumoto, M., Hatakeyama, S., and Seya, T. (2009). Riplet/RNF135, a RING finger protein, ubiquitinates RIG-I to promote interferon-beta induction during the early phase of viral infection. *The Journal of biological chemistry*, *284*(2), 807–17. doi: 10.1074/jbc.M804259200

Oshiumi, H., Miyashita, M., Inoue, N., Okabe, M., Matsumoto, M., and Seya, T. (2010). The ubiquitin ligase Riplet is essential for RIG-I-dependent innate immune responses to RNA virus infection. *Cell host & microbe*, *8*(6), 496–509. Elsevier Inc. doi: 10.1016/j.chom.2010.11.008

Paladino, P., Cummings, D. T. D., Noyce, R. S., and Mossman, K. L. (2006). The IFN-independent response to virus particle entry provides a first line of antiviral

defense that is independent of TLRs and retinoic acid-inducible gene I. *J. Immunol.*, (25).

Panne, D., Maniatis, T., and Harrison, S. C. (2004). Crystal structure of ATF-2/c-Jun and IRF-3 bound to the interferon-beta enhancer. *The EMBO journal*, *23*(22), 4384–93. doi: 10.1038/sj.emboj.7600453

Panne, D., Maniatis, T., and Harrison, S. C. (2007). An atomic model of the interferon-beta enhanceosome. *Cell*, *129*(6), 1111–23. doi: 10.1016/j.cell.2007.05.019

Paulmann, D., Magulski, T., Schwarz, R., Heitmann, L., Flehmig, B., Vallbracht, A., and Dotzauer, A. (2008). Hepatitis A virus protein 2B suppresses beta interferon (IFN) gene transcription by interfering with IFN regulatory factor 3 activation. *The Journal of general virology*, *89*(Pt 7), 1593–604. doi: 10.1099/vir.0.83521-0

Paun, A; Pitha, P. M. (2007). The IRF family, revisited. *Biochimie*, *89*(6-7), 744–53. doi: 10.1016/j.biochi.2007.01.014

Paz, S., Vilasco, M., Arguello, M., Sun, Q., Lacoste, J., Nguyen, T. L.-A., Zhao, T., et al. (2009). Ubiquitin-regulated recruitment of IkappaB kinase epsilon to the MAVS interferon signaling adapter. *Molecular and cellular biology*, *29*(12), 3401–12. doi: 10.1128/MCB.00880-08

Paz, S., Vilasco, M., Werden, S. J., Arguello, M., Joseph-Pillai, D., Zhao, T., Nguyen, T. L.-A., et al. (2011). A functional C-terminal TRAF3-binding site in MAVS participates in positive and negative regulation of the IFN antiviral response. *Cell research*, *21*(6), 895–910. Nature Publishing Group. doi: 10.1038/cr.2011.2

Perry, A. K., Chow, E. K., Goodnough, J. B., Yeh, W.-C., and Cheng, G. (2004). Differential requirement for TANK-binding kinase-1 in type I interferon responses to toll-like receptor activation and viral infection. *The Journal of experimental medicine*, *199*(12), 1651–8. doi: 10.1084/jem.20040528

Pinton, P., Giorgi, C., and Pandolfi, P. P. (2011). The role of PML in the control of apoptotic cell fate: a new key player at ER-mitochondria sites. *Cell death and differentiation*, *18*(9), 1450–6. Nature Publishing Group. doi: 10.1038/cdd.2011.31

Platanias, L. C. (2005). Mechanisms of type-I- and type-II-interferon-mediated signalling. *Nature reviews. Immunology*, *5*(5), 375–86. doi: 10.1038/nri1604

Poncet, D., Pauleau, A.-L., Szabadkai, G., Vozza, A., Scholz, S. R., Le Bras, M., Brière, J.-J., et al. (2006). Cytopathic effects of the cytomegalovirus-encoded apoptosis inhibitory protein vMIA. *The Journal of cell biology*, *174*(7), 985–96. doi: 10.1083/jcb.200604069

Pothlichet, J., Chignard, M., and Si-Tahar, M. (2008). Cutting Edge: Innate Immune Response Triggered by Influenza A Virus Is Negatively Regulated by SOCS1 and SOCS3 through a RIG-I/IFNAR1-Dependent Pathway. *The Journal of Immunology*, *180*(4), 2034–2038. doi: 10.4049/jimmunol.180.4.2034

Prinarakis, E., Chantzoura, E., Thanos, D., and Spyrou, G. (2008). S-glutathionylation of IRF3 regulates IRF3-CBP interaction and activation of the IFN beta pathway. *The EMBO journal*, *27*(6), 865–75. doi: 10.1038/emboj.2008.28

Pythoud, C., Rodrigo, W. W. S. I., Pasqual, G., Rothenberger, S., Martínez-Sobrido, L., de la Torre, J. C., and Kunz, S. (2012). Arenavirus nucleoprotein targets interferon

regulatory factor-activating kinase IKKε. *Journal of virology*, *86*(15), 7728–38. doi: 10.1128/JVI.00187-12

Qin, B. Y., Liu, C., Srinath, H., Lam, S. S., Correia, J. J., Derynck, R., and Lin, K. (2005). Crystal structure of IRF-3 in complex with CBP. *Structure (London, England : 1993)*, *13*(9), 1269–77. doi: 10.1016/j.str.2005.06.011

Qu, B., Qi, X., Wu, X., Liang, M., Li, C., Cardona, C. J., Xu, W., et al. (2012). Suppression of the interferon and NF-κB responses by severe fever with thrombocytopenia syndrome virus. *Journal of virology*, *86*(16), 8388–401. doi: 10.1128/JVI.00612-12

Rajput, A., Kovalenko, A., Bogdanov, K., Yang, S.-H., Kang, T.-B., Kim, J.-C., Du, J., et al. (2011). RIG-I RNA helicase activation of IRF3 transcription factor is negatively regulated by caspase-8-mediated cleavage of the RIP1 protein. *Immunity*, *34*(3), 340–51. Elsevier Inc. doi: 10.1016/j.immuni.2010.12.018

Ramos, H. J., and Gale, M. (2011). RIG-I like receptors and their signaling crosstalk in the regulation of antiviral immunity. *Current opinion in virology*, *1*(3), 167–76. Elsevier B.V. doi: 10.1016/j.coviro.2011.04.004

Rebsamen, M., Vazquez, J., Tardivel, A., Guarda, G., Curran, J., and Tschopp, J. (2011). NLRX1/NOD5 deficiency does not affect MAVS signalling. *Cell death and differentiation*, *18*(8), 1387. Nature Publishing Group. doi: 10.1038/cdd.2011.64

Reeves, R., and Beckerbauer, L. (2001). HMGI/Y proteins: flexible regulators of transcription and chromatin structure. *Biochimica et biophysica acta*, *1519*(1-2), 13–29.

Reis, L. F., Ruffner, H., Stark, G., Aguet, M., and Weissmann, C. (1994). Mice devoid of interferon regulatory factor 1 (IRF-1) show normal expression of type I interferon genes. *The EMBO journal*, *13*(20), 4798–806.

Ren, J., Kolli, D., Liu, T., Xu, R., Garofalo, R. P., Casola, A., and Bao, X. (2011). Human metapneumovirus inhibits IFN-β signaling by downregulating Jak1 and Tyk2 cellular levels. *PloS one*, *6*(9), e24496. doi: 10.1371/journal.pone.0024496

Ribeiro, D., Castro, I., Fahimi, H. D., and Schrader, M. (2012). Peroxisome morphology in pathology. *Histology and histopathology*, *27*(6), 661–76.

Ribet, D., and Cossart, P. (2010). Pathogen-mediated posttranslational modifications: A re-emerging field. *Cell*, *143*(5), 694–702. Elsevier Inc. doi: 10.1016/j.cell.2010.11.019

Richez, C., Barnetche, T., Miceli-Richard, C., Blanco, P., Moreau, J.-F., Rifkin, I., and Schaeverbeke, T. (2010). Role for interferon regulatory factors in autoimmunity. *Joint, bone, spine : revue du rhumatisme*, *77*(6), 525–31. doi: 10.1016/j.jbspin.2010.08.005

Rodriguez, J., Wang, L., and Horvath, C. (2003). Hendra virus V protein inhibits interferon signaling by preventing STAT1 and STAT2 nuclear accumulation. *Journal of virology*, *77*(21), 1–6. doi: 10.1128/JVI.77.21.11842

Ronco, L. V. L., Karpova, A. Y., Vidal, M., and Howley, P. M. (1998). Human papillomavirus 16 E6 oncoprotein binds to interferon regulatory factor-3 and inhibits its transcriptional activity. *Genes & Development*, *12*(13), 2061–2072. doi: 10.1101/gad.12.13.2061

Rothenfusser, S., Goutagny, N., DiPerna, G., Gong, M., Monks, B. G., Schoenemeyer, A., Yamamoto, M., et al. (2005). The RNA helicase Lgp2 inhibits TLR-independent sensing of viral replication by retinoic acid-inducible gene-I. *The Journal of Immunology*, (175), 5260–5268.

Saha, S. K., Pietras, E. M., He, J. Q., Kang, J. R., Liu, S.-Y., Oganesyan, G., Shahangian, A., et al. (2006). Regulation of antiviral responses by a direct and specific interaction between TRAF3 and Cardif. *The EMBO journal*, *25*(14), 3257–63. doi: 10.1038/sj.emboj.7601220

Saito, T., Hirai, R., Loo, Y.-M., Owen, D., Johnson, C. L., Sinha, S. C., Akira, S., et al. (2007). Regulation of innate antiviral defenses through a shared repressor domain in RIG-I and LGP2. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(2), 582–7. doi: 10.1073/pnas.0606699104

Saito, T., Owen, D. M., Jiang, F., Marcotrigiano, J., and Gale, M. (2008). Innate immunity induced by composition-dependent RIG-I recognition of hepatitis C virus RNA. *Nature*, *454*(7203), 523–7. doi: 10.1038/nature07106

Sanada, T., Takaesu, G., Mashima, R., Yoshida, R., Kobayashi, T., and Yoshimura, A. (2008). FLN29 deficiency reveals its negative regulatory role in the Toll-like receptor (TLR) and retinoic acid-inducible gene I (RIG-I)-like helicase signaling pathway. *The Journal of biological chemistry*, *283*(49), 33858–64. doi: 10.1074/jbc.M806923200

Sasai, M., Shingai, M., Funami, K., Yoneyama, M., Fujita, T., Matsumoto, M., and Seya, T. (2006). NAK-associated protein 1 participates in both the TLR3 and the cytoplasmic pathways in type I IFN induction. *Journal of immunology (Baltimore, Md. : 1950)*, *177*(12), 8676–83.

Sathish, N., Zhu, F. X., Golub, E. E., Liang, Q., and Yuan, Y. (2011). Mechanisms of autoinhibition of IRF-7 and a probable model for inactivation of IRF-7 by Kaposi's sarcoma-associated herpesvirus protein ORF45. *The Journal of biological chemistry*, *286*(1), 746–56. doi: 10.1074/jbc.M110.150920

Sato, M., Hata, N., Asagiri, M., Nakaya, T., Taniguchi, T., and Tanaka, N. (1998). Positive feedback regulation of type I IFN genes by the IFN-inducible transcription factor IRF-7. *FEBS letters*, *441*(1), 106–10.

Sato, M., Suemori, H., Hata, N., Asagiri, M., Ogasawara, K., Nakao, K., Nakaya, T., et al. (2000). Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-alpha/beta gene induction. *Immunity*, *13*(4), 539–48.

Sato, M., Tanaka, N., Hata, N., Oda, E., and Taniguchi, T. (1998). Involvement of the IRF family transcription factor IRF-3 in virus-induced activation of the IFN-beta gene. *FEBS letters*, *425*(1), 112–6.

Satoh, T., Kato, H., Kumagai, Y., Yoneyama, M., Sato, S., Matsushita, K., Tsujimura, T., et al. (2010). LGP2 is a positive regulator of RIG-I- and MDA5-mediated antiviral responses. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(4), 1512–7. doi: 10.1073/pnas.0912986107

Schlee, M., and Hartmann, G. (2010). The chase for the RIG-I ligand--recent advances. *Molecular therapy : the journal of the American Society of Gene Therapy*, *18*(7), 1254–62. Nature Publishing Group. doi: 10.1038/mt.2010.90

Schlee, M., Roth, A., Hornung, V., Hagmann, C. A., Wimmenauer, V., Barchet, W., Coch, C., et al. (2009). Recognition of 5' triphosphate by RIG-I helicase requires short blunt double-stranded RNA as contained in panhandle of negative-strand virus. *Immunity*, *31*(1), 25–34. Elsevier Ltd. doi: 10.1016/j.immuni.2009.05.008

Schmidt, A., and Schwerd, T. (2009). 5′-triphosphate RNA requires base-paired structures to activate antiviral signaling via RIG-I. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(29).

Schoggins, J. W., and Rice, C. M. (2011). Interferon-stimulated genes and their antiviral effector functions. *Current opinion in virology*, *1*(6), 519–25. Elsevier B.V. doi: 10.1016/j.coviro.2011.10.008

Schones, D. E., Cui, K., Cuddapah, S., Roh, T.-Y., Barski, A., Wang, Z., Wei, G., et al. (2008). Dynamic regulation of nucleosome positioning in the human genome. *Cell*, *132*(5), 887–98. doi: 10.1016/j.cell.2008.02.022

Schrader, M., and Fahimi, H. D. (2008). The peroxisome: still a mysterious organelle. *Histochemistry and cell biology*, *129*(4), 421–40. doi: 10.1007/s00418-008-0396-9

Schrader, M., and Yoon, Y. (2007). Mitochondria and peroxisomes: are the "big brother" and the "little sister" closer than assumed? *BioEssays : news and reviews in molecular, cellular and developmental biology*, *29*(11), 1105–14. doi: 10.1002/bies.20659

Schuhmann, K. M., Pfaller, C. K., and Conzelmann, K.-K. (2011). The measles virus V protein binds to p65 (RelA) to suppress NF-kappaB activity. *Journal of virology*, *85*(7), 3162–71. doi: 10.1128/JVI.02342-10

Sen, G. C., and Sarkar, S. N. (2007). The interferon-stimulated genes: targets of direct signaling by interferons, double-stranded RNA, and viruses. *Current topics in microbiology and immunology*, *316*, 233–50.

Seo, J. Y., Yaneva, R., and Cresswell, P. (2011). Viperin: a multifunctional, interferon-inducible protein that regulates virus replication. *Cell host & microbe*, *10*(6), 534–9. Elsevier Inc. doi: 10.1016/j.chom.2011.11.004

Seo, J. Y., Yaneva, R., Hinson, E. R., and Cresswell, P. (2011). Human cytomegalovirus directly induces the antiviral protein viperin to enhance infectivity. *Science*, *332*(6033), 1093–7. doi: 10.1126/science.1202007

Seth, R. B., Sun, L., Ea, C.-K., and Chen, Z. J. (2005). Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. *Cell*, *122*(5), 669–82. doi: 10.1016/j.cell.2005.08.012

Sharma, S., and Fitzgerald, K. a. (2010). Viral defense: it takes two MAVS to Tango. *Cell*, *141*(4), 570–2. doi: 10.1016/j.cell.2010.04.043

Sharma, S., TenOever, B. R., Grandvaux, N., Zhou, G.-P., Lin, R., and Hiscott, J. (2003). Triggering the interferon antiviral response through an IKK-related pathway. *Science*, *300*(5622), 1148–51. doi: 10.1126/science.1081315

Soares, F., Tattoli, I., Wortzman, M., Arnoult, D., Philpott, D., and Girardin, S. (2012). NLRX1 does not inhibit MAVS-dependent antiviral signalling. *Innate Immun.*, (19), 438–48.

Sohn, S.-Y., and Hearing, P. (2011). Adenovirus sequesters phosphorylated STAT1 at viral replication centers and inhibits STAT dephosphorylation. *Journal of virology*, *85*(15), 7555–62. doi: 10.1128/JVI.00513-11

Solis, M., Nakhaei, P., Jalalirad, M., Lacoste, J., Douville, R., Arguello, M., Zhao, T., et al. (2011). RIG-I-mediated antiviral signaling is inhibited in HIV-1 infection by a protease-mediated sequestration of RIG-I. *Journal of virology*, *85*(3), 1224–36. doi: 10.1128/JVI.01635-10

Song, T., Wei, C., Zheng, Z., Xu, Y., Cheng, X., Yuan, Y., Guan, K., et al. (2010). c-Abl tyrosine kinase interacts with MAVS and regulates innate immune response. *FEBS letters*, *584*(1), 33–8. Federation of European Biochemical Societies. doi: 10.1016/j.febslet.2009.11.025

Sumpter, R., Loo, Y., Foy, E., and Li, K. (2005). Regulating intracellular antiviral defense and permissiveness to hepatitis C virus RNA replication through a cellular RNA helicase, RIG-I. *Journal of Virology*, *79*(5), 2689–2699. doi: 10.1128/JVI.79.5.2689

Sun, L., Xing, Y., Chen, X., Zheng, Y., Yang, Y., Nichols, D. B., Clementz, M. a, et al. (2012). Coronavirus papain-like proteases negatively regulate antiviral innate immune response through disruption of STING-mediated signaling. *PloS one*, *7*(2), e30802. doi: 10.1371/journal.pone.0030802

Sun, W., Li, Y., Chen, L., Chen, H., You, F., Zhou, X., Zhou, Y., et al. (2009). ERIS, an endoplasmic reticulum IFN stimulator, activates innate immune signaling through dimerization. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(21), 8653–8. doi: 10.1073/pnas.0900850106

Sun, Y. W. (1997). Rig-I, a human homolog gene of RNA helicase, is induced by retinoic acid during the differentiation of acute promyelocytic leukemia cell. *Thesis, Shanghai Institute of Hematology, Shanghai Second Medical University*.

Sun, Z., Ren, H., Liu, Y., Teeling, J. L., and Gu, J. (2011). Phosphorylation of RIG-I by casein kinase II inhibits its antiviral response. *Journal of virology*, *85*(2), 1036–47. doi: 10.1128/JVI.01734-10

Suthar, M. S., Ramos, H. J., Brassil, M. M., Netland, J., Chappell, C. P., Blahnik, G., McMillan, A., et al. (2012). The RIG-I-like receptor LGP2 controls CD8(+) T cell survival and fitness. *Immunity*, *37*(2), 235–48. doi: 10.1016/j.immuni.2012.07.004

Takahashi, K., Kawai, T., and Kumar, H. (2006). Roles of caspase-8 and caspase-10 in innate immune responses to double-stranded RNA. *The Journal of Immunology*, (176), 4520–4524.

Takahasi, K., Kumeta, H., Tsuduki, N., Narita, R., Shigemoto, T., Hirai, R., Yoneyama, M., et al. (2009). Solution structures of cytosolic RNA sensor MDA5 and LGP2 C-terminal domains: identification of the RNA recognition loop in RIG-I-like receptors. *The Journal of biological chemistry*, *284*(26), 17465–74. doi: 10.1074/jbc.M109.007179

Takahasi, K., Yoneyama, M., Nishihori, T., Hirai, R., Kumeta, H., Narita, R., Gale, M., et al. (2008). Nonself RNA-sensing mechanism of RIG-I helicase and activation of antiviral immune responses. *Molecular cell*, *29*(4), 428–40. doi: 10.1016/j.molcel.2007.11.028

Takaoka, A., Wang, Z., Choi, M. K., Yanai, H., Negishi, H., Ban, T., Lu, Y., et al. (2007). DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response. *Nature*, *448*(7152), 501–5. doi: 10.1038/nature06013

Takeuchi, O., and Akira, S. (2008). MDA5/RIG-I and virus recognition. *Current opinion in immunology*, *20*(1), 17–22. doi: 10.1016/j.coi.2008.01.002

Tal, M. C., Sasai, M., Lee, H. K., Yordy, B., Shadel, G. S., and Iwasaki, A. (2009). Absence of autophagy results in reactive oxygen species-dependent amplification of RLR signaling. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(8), 2770–5. doi: 10.1073/pnas.0807694106

Talaro, K. (2002). *Foundations in Microbiology* (4th ed.). The McGraw−Hill Companies.

Talon, J., Horvath, C. M., Polley, R., Basler, C. F., Muster, T., Palese, P., and Garcia-Sastre, A. (2000). Activation of Interferon Regulatory Factor 3 Is Inhibited by the Influenza A Virus NS1 Protein. *Journal of Virology*, *74*(17), 7989–7996. doi: 10.1128/JVI.74.17.7989-7996.2000

Tan, J. C. G., Avdic, S., Cao, J. Z., Mocarski, E. S., White, K. L., Abendroth, A., and Slobedman, B. (2011). Inhibition of 2’,5'-oligoadenylate synthetase expression and function by the human cytomegalovirus ORF94 gene product. *Journal of virology*, *85*(11), 5696–700. doi: 10.1128/JVI.02463-10

Tang, E. D., and Wang, C.-Y. (2009). MAVS self-association mediates antiviral innate immune signaling. *Journal of virology*, *83*(8), 3420–8. doi: 10.1128/JVI.02623-08

Temin, H. M. (1985). Review Reverse Transcription in the Eukaryotic Genome: Retroviruses, Pararetroviruses, Retrotransposons, and Retrotranscripts. *Mollecular Biology and Evolution*, *2*(6), 455–468.

Tenoever, B. R., Ng, S.-L., Chua, M. a, McWhirter, S. M., García-Sastre, A., and Maniatis, T. (2007). Multiple functions of the IKK-related kinase IKKepsilon in interferon-mediated antiviral immunity. *Science*, *315*(5816), 1274–8. doi: 10.1126/science.1136567

Thanos, D., and Maniatis, T. (1995). Virus induction of human IFN beta gene expression requires the assembly of an enhanceosome. *Cell*, *83*(7), 1091–100.

The Science Picture Company. (2014). The Science Picture Company. Retrieved March 20, 2014, from http://www.sciencepicturecompany.com/

Tu, Y.-C., Yu, C.-Y., Liang, J.-J., Lin, E., Liao, C.-L., and Lin, Y.-L. (2012). Blocking double-stranded RNA-activated protein kinase PKR by Japanese encephalitis virus nonstructural protein 2A. *Journal of virology*, *86*(19), 10347–58. doi: 10.1128/JVI.00525-12

Ulane, C. M., and Horvath, C. M. (2002). Paramyxoviruses SV5 and HPIV2 Assemble STAT Protein Ubiquitin Ligase Complexes from Cellular Components. *Virology*, *304*(2), 160–166. doi: 10.1006/viro.2002.1773

Unterholzner, L., Sumner, R. P., Baran, M., Ren, H., Mansur, D. S., Bourke, N. M., Randow, F., et al. (2011). Vaccinia virus protein C6 is a virulence factor that binds TBK-1 adaptor proteins and inhibits activation of IRF3 and IRF7. *PLoS pathogens*, *7*(9), e1002247. doi: 10.1371/journal.ppat.1002247

Uzri, D., and Gehrke, L. (2009). Nucleotide sequences and modifications that determine RIG-I/RNA binding and signaling activities. *Journal of virology*, *83*(9), 4174–84. doi: 10.1128/JVI.02449-08

Vandevenne, P., Lebrun, M., El Mjiyad, N., Ote, I., Di Valentin, E., Habraken, Y., Dortu, E., et al. (2011). The varicella-zoster virus ORF47 kinase interferes with host innate immune response by inhibiting the activation of IRF3. *PloS one*, *6*(2), e16870. doi: 10.1371/journal.pone.0016870

Varga, Z. T., Grant, A., Manicassamy, B., and Palese, P. (2012). Influenza virus protein PB1-F2 inhibits the induction of type I interferon by binding to MAVS and decreasing mitochondrial membrane potential. *Journal of virology*, *86*(16), 8359–66. doi: 10.1128/JVI.01122-12

Venkataraman, T., Valdes, M., Elsby, R., Kakuta, S., Caceres, G., Saijo, S., Iwakura, Y., et al. (2007). Loss of DExD/H box RNA helicase LGP2 manifests disparate antiviral responses. *The Journal of immunology*, *178*(10), 6444–55.

ViralZone. (2014). The Baltimore classification of viruses. Retrieved March 17, 2014, from http://viralzone.expasy.org/all_by_species/254.html

Visvanathan, K. V, and Goodbourn, S. (1989). Double-stranded RNA activates binding of NF-kappa B to an inducible element in the human beta-interferon promoter. *The EMBO journal*, *8*(4), 1129–38.

Vitour, D., Dabo, S., Ahmadi Pour, M., Vilasco, M., Vidalain, P.-O., Jacob, Y., Mezel-Lemoine, M., et al. (2009). Polo-like kinase 1 (PLK1) regulates interferon (IFN) induction by MAVS. *The Journal of biological chemistry*, *284*(33), 21797–809. doi: 10.1074/jbc.M109.018275

Wang, D., Fang, L., Li, K., Zhong, H., Fan, J., Ouyang, C., Zhang, H., et al. (2012). Foot-and-Mouth Disease Virus 3C Protease Cleaves NEMO To Impair Innate Immune Signaling. *Journal of Virology*, *86*(17), 9311–9322. doi: 10.1128/JVI.00722-12

Wang, G., Chen, G., Zheng, D., Cheng, G., and Tang, H. (2011). PLP2 of mouse hepatitis virus A59 (MHV-A59) targets TBK1 to negatively regulate cellular type I interferon signaling pathway. *PloS one*, *6*(2), e17192. doi: 10.1371/journal.pone.0017192

Wang, P., Arjona, A., Zhang, Y., Sultana, H., Dai, J., Yang, L., LeBlanc, P. M., et al. (2010). Caspase-12 controls West Nile virus infection via the viral RNA receptor RIG-I. *Nature immunology*, *11*(10), 912–9. doi: 10.1038/ni.1933

Wang, P., Yang, L., Cheng, G., Yang, G., Xu, Z., You, F., Sun, Q., et al. (2013). UBXN1 interferes with Rig-I-like receptor-mediated antiviral immune response by targeting MAVS. *Cell reports*, *3*(4), 1057–70. The Authors. doi: 10.1016/j.celrep.2013.02.027

Wang, Y., Tong, X., Omoregie, E. S., Liu, W., Meng, S., and Ye, X. (2012). Tetraspanin 6 (TSPAN6) negatively regulates retinoic acid-inducible gene I-like receptor-mediated immune signaling in a ubiquitination-dependent manner. *The Journal of biological chemistry*, *287*(41), 34626–34. doi: 10.1074/jbc.M112.390401

Wang, Y., Tong, X., and Ye, X. (2012). Ndfip1 negatively regulates RIG-I-dependent immune signaling by enhancing E3 ligase Smurf1-mediated MAVS degradation.

Journal of immunology (Baltimore, Md. : 1950), *189*(11), 5304–13. doi: 10.4049/jimmunol.1201445

Wathelet, M. G., Lin, C. H., Parekh, B. S., Ronco, L. V, Howley, P. M., and Maniatis, T. (1998). Virus infection induces the assembly of coordinately activated transcription factors on the IFN-beta enhancer in vivo. *Molecular cell*, *1*(4), 507–18.

Weaver, B. K., Kumar, K. P., and Reich, N. C. (1998). Interferon regulatory factor 3 and CREB-binding protein/p300 are subunits of double-stranded RNA-activated transcription factor DRAF1. *Molecular and cellular biology*, *18*(3), 1359–68.

Wen, C., Yan, Z., Yang, X., Guan, K., Xu, C., Song, T., Zheng, Z., et al. (2012). Identification of tyrosine-9 of MAVS as critical target for inducible phosphorylation that determines activation. *PloS one*, *7*(7), e41687. doi: 10.1371/journal.pone.0041687

West, A. P., Shadel, G. S., and Ghosh, S. (2011). Mitochondria in innate immune responses. *Nature reviews. Immunology*, *11*(6), 389–402. Nature Publishing Group. doi: 10.1038/nri2975

White, J. P., Reineke, L. C., and Lloyd, R. E. (2011). Poliovirus switches to an eIF2-independent mode of translation during infection. *Journal of virology*, *85*(17), 8884–93. doi: 10.1128/JVI.00792-11

Wilkins, C., and Gale, M. (2010). Recognition of viruses by cytoplasmic sensors. *Current opinion in immunology*, *22*(1), 41–7. Elsevier Ltd. doi: 10.1016/j.coi.2009.12.003

Xu, L., Xiao, N., Liu, F., Ren, H., and Gu, J. (2009). Inhibition of RIG-I and MDA5-dependent antiviral response by gC1qR at mitochondria. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(5), 1530–5. doi: 10.1073/pnas.0811029106

Xu, L.-G., Wang, Y.-Y., Han, K.-J., Li, L.-Y., Zhai, Z., and Shu, H.-B. (2005). VISA is an adapter protein required for virus-triggered IFN-beta signaling. *Molecular cell*, *19*(6), 727–40. doi: 10.1016/j.molcel.2005.08.014

Yang, K., Shi, H., Qi, R., and Sun, S. (2006). Hsp90 regulates activation of interferon regulatory factor 3 and TBK-1 stabilization in Sendai virus-infected cells. *Molecular Biology of the Cell*, *17*(March), 1461–1471. doi: 10.1091/mbc.E05

Yang, P., An, H., Liu, X., Wen, M., Zheng, Y., Rui, Y., and Cao, X. (2010). The cytosolic nucleic acid sensor LRRFIP1 mediates the production of type I interferon via a beta-catenin-dependent pathway. *Nature immunology*, *11*(6), 487–94. Nature Publishing Group. doi: 10.1038/ni.1876

Ye, J., and Maniatis, T. (2011). Negative regulation of interferon-β gene expression during acute and persistent virus infections. *PloS one*, *6*(6), e20681. doi: 10.1371/journal.pone.0020681

Yie, J., Liang, S., Merika, M., and Thanos, D. (1997). Intra- and intermolecular cooperative binding of high-mobility-group protein I(Y) to the beta-interferon promoter. *Molecular and cellular biology*, *17*(7), 3649–62.

Yie, J., Merika, M., Munshi, N., Chen, G., and Thanos, D. (1999). The role of HMG I(Y) in the assembly and function of the IFN-beta enhanceosome. *The EMBO journal*, *18*(11), 3074–89. doi: 10.1093/emboj/18.11.3074

Yokota, S., Saito, H., Kubota, T., Yokosawa, N., Amano, K., and Fujii, N. (2003). Measles virus suppresses interferon-alpha signaling pathway: suppression of Jak1 phosphorylation and association of viral accessory proteins, C and V, with interferon-alpha receptor complex. *Virology*, *306*(1), 135–46.

Yoneyama, M., and Fujita, T. (2008). Structural mechanism of RNA recognition by the RIG-I-like receptors. *Immunity*, *29*(2), 178–81. doi: 10.1016/j.immuni.2008.07.009

Yoneyama, M., Kikuchi, M., Matsumoto, K., Imaizumi, T., Miyagishi, M., Taira, K., Foy, E., et al. (2005). Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. *The Journal of immunology*, *175*(5), 2851–8.

Yoneyama, M., Kikuchi, M., Natsukawa, T., Shinobu, N., Imaizumi, T., Miyagishi, M., Taira, K., et al. (2004). The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nature immunology*, *5*(7), 730–7. doi: 10.1038/ni1087

Yoneyama, M., Suhara, W., Fukuhara, Y., Fukuda, M., Nishida, E., and Fujita, T. (1998). Direct triggering of the type I interferon system by virus infection: activation of a transcription factor complex containing IRF-3 and CBP/p300. *The EMBO journal*, *17*(4), 1087–95. doi: 10.1093/emboj/17.4.1087

Yoshida, R., Takaesu, G., Yoshida, H., Okamoto, F., Yoshioka, T., Choi, Y., Akira, S., et al. (2008). TRAF6 and MEKK1 play a pivotal role in the RIG-I-like helicase antiviral pathway. *The Journal of biological chemistry*, *283*(52), 36211–20. doi: 10.1074/jbc.M806576200

You, F., Sun, H., Zhou, X., Sun, W., Liang, S., Zhai, Z., and Jiang, Z. (2009). PCBP2 mediates degradation of the adaptor MAVS via the HECT ubiquitin ligase AIP4. *Nat. Immunol .*, *10*, 1300–1308.

You, F., Sun, H., Zhou, X., Sun, W., Liang, S., Zhai, Z., and Jiang, Z. (2009). PCBP2 mediates degradation of the adaptor MAVS via the HECT ubiquitin ligase AIP4. *Nature immunology*, *10*(12), 1300–8. Nature Publishing Group. doi: 10.1038/ni.1815

Young, J., Hoogenraad, N., and Hartl, F. (2003). Molecular chaperones Hsp90 and Hsp70 deliver preproteins to the mitochondrial import receptor Tom70. *Cell*, *112*, 41–50.

Yu, C.-Y., Chang, T.-H., Liang, J.-J., Chiang, R.-L., Lee, Y.-L., Liao, C.-L., and Lin, Y.-L. (2012). Dengue virus targets the adaptor protein MITA to subvert host innate immunity. *PLoS pathogens*, *8*(6), e1002780. doi: 10.1371/journal.ppat.1002780

Zeng, W., Sun, L., Jiang, X., Chen, X., Hou, F., Adhikari, A., Xu, M., et al. (2010). Reconstitution of the RIG-I pathway reveals a signaling role of unanchored polyubiquitin chains in innate immunity. *Cell*, *141*(2), 315–30. Elsevier Ltd. doi: 10.1016/j.cell.2010.03.029

Zhang, M., Wu, X., Lee, A. J., Jin, W., Chang, M., Wright, A., Imaizumi, T., et al. (2008). Regulation of IkappaB kinase-related kinases and antiviral responses by tumor suppressor CYLD. *The Journal of biological chemistry*, *283*(27), 18621–6. doi: 10.1074/jbc.M801451200

Zhao, L., Jha, B. K., Wu, A., Elliott, R., Ziebuhr, J., Gorbalenya, A. E., Silverman, R. H., et al. (2012). Antagonism of the interferon-induced OAS-RNase L pathway by

murine coronavirus ns2 protein is required for virus replication and liver pathology. *Cell host & microbe*, *11*(6), 607–16. Elsevier Inc. doi: 10.1016/j.chom.2012.04.011

Zhao, T., Yang, L., Sun, Q., Arguello, M., Ballard, D. W., Hiscott, J., and Lin, R. (2007). The NEMO adaptor bridges the nuclear factor-kappaB and interferon regulatory factor signaling pathways. *Nature immunology*, *8*(6), 592–600. doi: 10.1038/ni1465

Zhao, Y., Sun, X., Nie, X., Sun, L., Tang, T.-S., Chen, D., and Sun, Q. (2012). COX5B regulates MAVS-mediated antiviral signaling through interaction with ATG5 and repressing ROS production. *PLoS pathogens*, *8*(12), e1003086. doi: 10.1371/journal.ppat.1003086

Zhong, B., Yang, Y., Li, S., Wang, Y.-Y., Li, Y., Diao, F., Lei, C., et al. (2008). The adaptor protein MITA links virus-sensing receptors to IRF3 transcription factor activation. *Immunity*, *29*(4), 538–50. Elsevier Inc. doi: 10.1016/j.immuni.2008.09.003

Zhong, B., Zhang, Y., Tan, B., Liu, T.-T., Wang, Y.-Y., and Shu, H.-B. (2010). The E3 ubiquitin ligase RNF5 targets virus-induced signaling adaptor for ubiquitination and degradation. *Journal of immunology (Baltimore, Md.: 1950)*, *184*(11), 6249–55. doi: 10.4049/jimmunol.0903748

Zhou, X., You, F., Chen, H., and Jiang, Z. (2012). Poly(C)-binding protein 1 (PCBP1) mediates housekeeping degradation of mitochondrial antiviral signaling (MAVS). *Cell research*, *22*(4), 717–27. Nature Publishing Group. doi: 10.1038/cr.2011.184

Zhu, H., Zheng, C., Xing, J., Wang, S., Li, S., Lin, R., and Mossman, K. L. (2011). Varicella-zoster virus immediate-early protein ORF61 abrogates the IRF3-mediated innate immune response through degradation of activated IRF3. *Journal of virology*, *85*(21), 11079–89. doi: 10.1128/JVI.05098-11

Zhu, Z., Zhang, X., Wang, G., and Zheng, H. (2014). The Laboratory of Genetics and Physiology 2: Emerging Insights into the Controversial Functions of This RIG-I-Like Receptor. *BioMed research international*, *2014*, 960190. doi: 10.1155/2014/960190

Zygote Media Group Inc. (2014). 3DScience.com. Retrieved March 20, 2014, from http://www.3dscience.com/