Chapter 1

General Introduction

UNDERSTANDING VIRUS-HOST INTERPLAY

Despite their limited size, viruses can have a massive impact on their hosts on both the individual and population level. For example, one can think of the devastating effects on the human body of Ebola virus infection, the worldwide societal and economic unrest caused by the SARS-coronavirus pandemic in 2002-2003, or the millions of deaths associated with the Spanish flu pandemic in the early 20th century. These events become even more fascinating when taking into consideration the nature of viral particles, essentially being lifeless entities. Viruses are incapable of replicating outside of a cellular context and thus depend entirely on interactions with their host for their reproduction. These interactions take place at numerous and very diverse steps in the viral "life cycle". For example, they range from the use of cellular receptors for viral entry into the cell to hitching a ride on the cellular secretory pathway to facilitate egress, and from viral hijacking of the cellular translation machinery to counteracting cellular antiviral defence systems. A complete understanding of viral biology, if ever attainable, therefore relies for a large part on unravelling the interplay between viruses and their hosts.

In the case of positive-strand (+) RNA viruses, virus-encoded proteases provide an excellent tool to manipulate the intracellular environment. Especially mammalian +RNA viruses commonly express (part of) their proteome in the form of large polyproteins that are co- and posttranslationally cleaved into their respective functional subunits by internal protease domains. Interestingly, many virus-encoded proteases have been found to exert a dual function, targeting both the viral polyprotein and host proteins. For example, the hepatitis C virus main protease, which is critical for replication because of its role in polyprotein maturation, has been shown to cleave and thereby inactivate the innate immune signalling adaptor protein MAVS (1, 2). To broaden our understanding of viral protease-mediated manipulation of the intracellular environment, this thesis focuses on elucidating the interaction between a protease encoded by a particular group of +RNA viruses, the arteriviruses (order *Nidovirales*), and the cellular ubiquitin system, both of which will be introduced in detail below.

THE NIDOVIRUS ORDER

Taxonomy

The order *Nidovirales* includes a collection of animal viruses that are characterized by a common expression strategy and genome organization, and by the presumed common ancestry of key replicative enzymes (for reviews, see (3, 4)). Its name derives from the nested set (the Latin *nidus* means nest) of subgenomic mRNAs that forms a com-

mon feature of nidovirus genome expression (5). At the moment, the nidovirus order is subdivided into four families: the arteriviruses, coronaviruses, mesoniviruses, and roniviruses (4, 6). The arterivirus family currently consists of one genus and includes four species: the family prototype equine arteritis virus (EAV), porcine reproductive and respiratory syndrome virus (PRRSV), lactate dehydrogenase-elevating virus (LDV), and simian hemorrhagic fever virus (SHFV) (7). In nature, these viruses each display a strikingly narrow host range by infecting equids, swine, mice, or monkeys, respectively. Two genotypes of PRRSV (type I and II) are currently recognized and these show such low levels of sequence similarity (60-70% at the nucleotide sequence level (8)) that they will likely be classified as separate species upon future taxonomic revisions. Furthermore, the recently identified wobbly possum disease virus (WPDV) (9) and several newly recognized monkey viruses distantly related to SHFV (10) are now tentatively placed within the arterivirus family.

The coronavirus family has a more complicated internal taxonomic structure and, contrary to the arteriviruses, does contain a number of human viruses. It includes two subfamilies, *coronavirinae* and *torovirinae*, the former including a total of four *genera*: the alpha-, beta-, gamma-, and deltacoronaviruses (11). Coronaviruses infect a wide variety of animals, including for example white bream, chicken, beluga whales, bats, mice, swine, and humans. Whereas human coronaviruses, such as NL63 and 229E, are responsible for a percentage of (often mild) cases of common cold, two zoonotic betacoronaviruses cause much more severe disease in humans. These are the severe acute respiratory syndrome-associated coronavirus (SARS-CoV) (12-14) and the recently emerged Middle East respiratory syndrome coronavirus (MERS-CoV) (15). Finally, the mesonivirus family consists of several species of insect virus (16-18) and the ronivirus family consists of two prawn virus species (19-21).

The work described in this thesis mainly focuses on EAV, which is the prototype arterivirus and has been studied for a considerable number of years since its discovery in the late 1950s (22, 23). Since PRRSV is considered an economically highly relevant pathogen with a major impact on swine farming industries worldwide (24), and because some of the work described in this thesis relates to possibilities for its improved control, it will also be included in this introduction. LDV, SHFV, and the novel arteriviruses will not be discussed further here (for a recent review, see (25)). Instead, some attention will be given to the distantly related SARS- and MERS-coronaviruses because of their relevance for public health and the functional similarities with the arteriviruses in terms of the interactions of their proteases with the cellular ubiquitin system.

Pathogenesis and impact

Although the majority of infections with EAV remain subclinical, outbreaks of the associated disease equine viral arteritis (EVA) are sometimes observed (26-29). The symptoms characterizing this disease are inflammation of the small muscular arteries (arteritis), fever, depression, anorexia, and oedema in adult horses, abortion in pregnant mares, and interstitial pneumonia in young foals (for a recent review, see (30)). An important factor in the epidemiology and spread of EAV appears to be the occurrence of persistent infection in the ampulla of the reproductive tract of breeding stallions, which allows for venereal transmission of the virus through infected semen (31). Alternatively, infection can occur via the respiratory tract through inhalation of virus-containing aerosols from respiratory secretions or urine (32). Prevention of EVA mainly relies on vaccination (33), for which currently two types of vaccine are commercially available: a modified live virus (MLV) and an inactivated virus vaccine called ARVAC and Artervac, respectively (both marketed by Zoetis, NJ, USA). The former is considered more effective than the latter vaccine type, but some issues pertaining to the use of the MLV do exist. Firstly, it is not recommended for use in pregnant mares during the last two months of gestation (32). In addition, and more importantly, even though vaccination with the MLV prevents the onset of clinical EVA, limited replication of field strains in vaccinated animals, combined with occasional shedding of infectious virus, has been observed (34). Since the spread of EAV potentially has a profound impact on horse breeding and performance industries, due to for example the loss of foals, decreased value of carrier stallions, and cancellation of equestrian events (33), its effective control is of significant importance. A combination of vaccination and prevention strategies based on screening for and isolation of infected animals currently appear to be effective in containing EAV, although there is certainly room for improvement of the efficacy and safety of the available vaccines against this virus.

The syndrome associated with PRRSV infection is characterized by abortions in pregnant sows and severe respiratory illness in young pigs (for a review, see (35)). Especially the emergence of highly virulent strains of PRRSV in China in 2006 is associated with very severe illness and high mortality rates (36-38). In addition, PRRSV infection is characterized by a long-lasting viraemia, caused by persistent infection of the tonsils, lungs, and lymphoid organs (39-41). Importantly, PRRSV causes considerable losses to swine farming industries worldwide, with estimates of at least half a billion dollar per year in the United States alone (24). Currently, a number of PRRSV vaccines are commercially available, of which the MLVs are considered to be most effective. However, like the EAV MLV, these vaccines appear to only avert the clinical manifestation of subsequent PRRSV infection without completely preventing replication of field strains (for reviews, see (42, 43)). In addition, these vaccines offer little to no protection against heterologous strains, even those belonging to the same

genotype. Taken together, there is a great need for improved PRRSV vaccines, preferably in the form of novel MLVs that provide better protection against heterologous field strains than currently available vaccines.

Until 2002, coronaviruses were only known to cause mild respiratory illness upon infection of otherwise healthy humans. However, that year witnessed the emergence of the zoonotic SARS-CoV as a novel and highly pathogenic human pathogen (12-14, 44). It quickly spread across the globe and was responsible for the death of approximately 800 people worldwide, resulting in a mortality rate of about 10% (for a review, see (45)). Although no vaccines have been approved for clinical use to date, the pandemic was quickly contained using strict quarantine measures (for reviews, see (45-47)). More recently, in 2012, another zoonotic coronavirus emerged which was later named Middle East respiratory syndrome coronavirus (MERS-CoV) after its geographical origin and associated pathology (15). As of July 2014, more than 800 laboratory-confirmed MERS cases had been reported to the World Health Organisation, resulting in 289 deaths (source: www.who.org). Although this novel addition to the coronavirus family appears to spread less rapidly than the SARS-CoV, it does cause severe pathology with high mortality rates (currently about 35%) in susceptible individuals and it remains to be seen how it will evolve in the near future.

General features of nidovirus replication

Nidoviruses are enveloped +RNA viruses with a non-segmented genome ranging in size from approximately 13 (EAV) to 30 (SARS- and MERS-CoVs) kilobases (kb). Upon infection, the viral RNA is released into the cytoplasm where the two largest open reading frames (ORF1a and ORF1b), covering the 5'-proximal two-thirds to three-quarters of the viral genome, are immediately translated into two overlapping replicase polyproteins: pp1a and pp1ab (**Figure I-1**). The latter is produced via a -1 programmed ribosomal frameshift (PRF), which occurs with a frequency of approximately 15-20% when tested in a reporter system for EAV (48, 49). The replicase polyproteins consist of the viral nonstructural proteins (nsps), which are released via extensive autoproteolytic processing (discussed below) and include key replicative enzymes such as the RNA-dependent RNA polymerase and helicase. Together, the nsps form the membrane-bound replication and transcription complex (RTC) which is responsible for the replication of viral genomic RNA and the transcription of subgenomic mRNAs (5). This complex localizes to endoplasmic reticulum (ER)-derived membrane structures consisting predominantly of double-membrane vesicles (DMVs) (50, 51) whose induction, in the case of EAV, can be mimicked by the combined expression of nsp2 and nsp3 (52, 53). The nidovirus structural proteins (nucleocapsid and envelope proteins) and, in the case of coronaviruses, several group-specific accessory proteins are translated from a nested set of subgenomic mRNAs that derive from the 3'-proximal

region of the viral genome. Encapsidation of the viral genome is followed by budding into the smooth ER, ERGIC or Golgi complex, where the viral envelope proteins appear to be retained (54, 55). Newly formed viral particles are then probably transported to the plasma membrane via the secretory pathway and released from the cell.

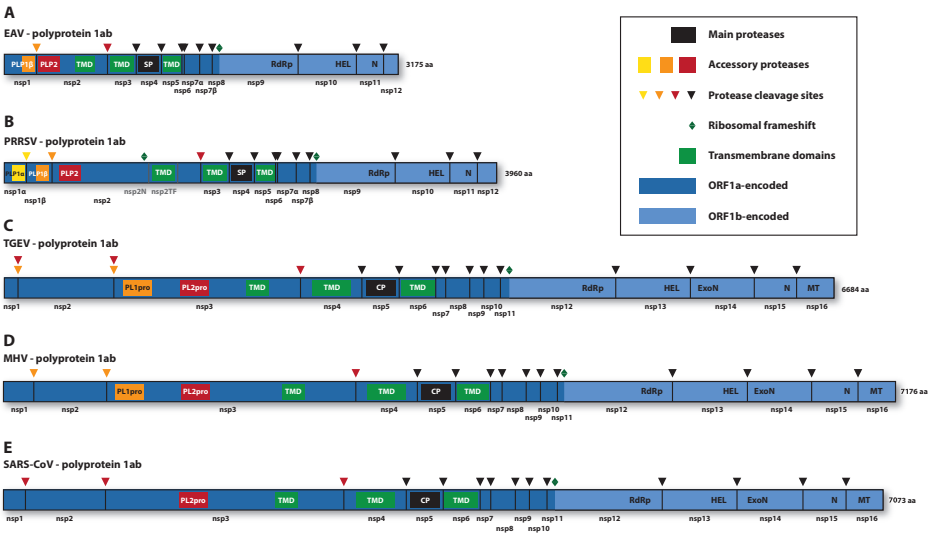

Figure I-1: Nidovirus replicase polyproteins. Schematic representations of the replicase polyproteins (pp1ab) of **(A)** equine arteritis virus (EAV), **(B)** porcine respiratory and reproductive syndrome virus (PRRSV), **(C)** transmissible gastroenteritis virus (TGEV), **(D)** mouse hepatitis virus (MHV), and **(E)** severe acute respiratory syndrome-associated coronavirus (SARS-CoV). Key replicative enzymes (RNA-dependent RNA polymerase and helicase), ribosomal frameshift sites, (putative) transmembrane domains, main and accessory protease domains, and protease cleavage sites are indicated (see also in-figure legend). Proteins are drawn to scale, individual domains are not. **Abbreviations:** aa, amino acids; CP, cysteine protease; ExoN, exonuclease; HEL, helicase; MT, methyltransferase; N, uridylate-specific endoribonuclease (NendoU); nsp, nonstructural protein; nsp2N, truncated protein encompassing the N-terminal part of nsp2; nsp2TF, transframe fusion product of nsp2; ORF, open reading frame; PLP/PLpro, papain-like protease; pp, polyprotein; RdRp, RNA-dependent RNA polymerase; SP, serine protease; TMD, transmembrane domain.

The essential role of nidovirus proteases

As mentioned above, the nidovirus nsps are expressed as two large and partially overlapping polyproteins (**Figure I-1**). Their co- and posttranslational cleavage is strictly ordered and is presumed to be entirely mediated by internal protease domains. Each nidovirus replicase gene encodes one main protease domain, which is a chymotrypsin-like serine protease (SP) in arteriviruses and *torovirinae* and a chymotrypsin-like cysteine protease (CP) in *coronavirinae,* mesoni- and roniviruses (3, 18, 56). In addition, the arteri- and coronaviruses encode one or more accessory protease domains, which

are papain-like cysteine proteases designated PLP or PLpro, respectively. Especially the proteolytic processing of the EAV replicase polyproteins has been extensively studied, and will be discussed here as an example. The first cleavage event in EAV polyprotein maturation is mediated by the PLP1β domain located in nsp1, which co-translationally cleaves the junction between nsp1 and nsp2 and thereby releases nsp1 from the nascent polyprotein (57, 58). The next cleavage is mediated by the PLP2 domain, which is located in the N-terminal region of nsp2 and is responsible for the processing of the nsp2|nsp3 junction (59). Finally, the nsp4 SP domain is responsible for the remaining cleavages in pp1a and pp1ab (58, 60-62), in which the sequence and outcome of cleavages in the nsp3-8 region can follow either one of two mutually exclusive pathways (63). In the majority of polyproteins, processing starts with cleavage of the nsp4|nsp5 junction. This is then followed by cleavage of the remaining sites, except for the nsp5|nsp6 and nsp6|nsp7 junctions, which remain unprocessed. This scheme is referred to as the major processing pathway. Alternatively, in the minor processing pathway, the nsp4|nsp5 junction is not cleaved. Instead, processing takes place at all of the other junctions, including the nsp5|nsp6 and nsp6|nsp7 cleavage sites. It appears that cleavage of the nsp4|nsp5 junction in the major processing pathway strongly depends on the presence of cleaved nsp2, which thus seems to act as a co-factor to the main protease during this particular cleavage event (63). Importantly, viral replication heavily depends on the correct and timely processing of the replicase polyproteins and mutagenesis of the active site residues or cleavage sites of the main and accessory arterivirus proteases has profound and generally lethal consequences for viral replication (53, 61, 62, 64).

A BRIEF INTRODUCTION TO UBIQUITIN

Ubiquitination and linkage types

Ubiquitin is a small (8.5-kDa) protein with a characteristic β-grasp fold. It can be covalently attached to lysine side-chains or the N terminus of target proteins via its C-terminal glycine residue. This process, referred to as ubiquitination, occurs via an enzymatic cascade that is mediated by an E1 activating enzyme, an E2 conjugating enzyme, and an E3 ligase. Ubiquitination plays an important regulatory role in a wide array of cellular processes, including for example the proteasomal degradation pathway, the DNA damage response, autophagy, and innate immunity (for reviews, see (65-68)). In order to modulate such a variety of processes, ubiquitination can occur in many different forms (for a recent review, see (69)). In its simplest form, referred to as monoubiquitination, a single ubiquitin molecule is attached to one lysine residue in a target protein (**Figure I-2A**). During multimonoubiquitination, several ubiquitin

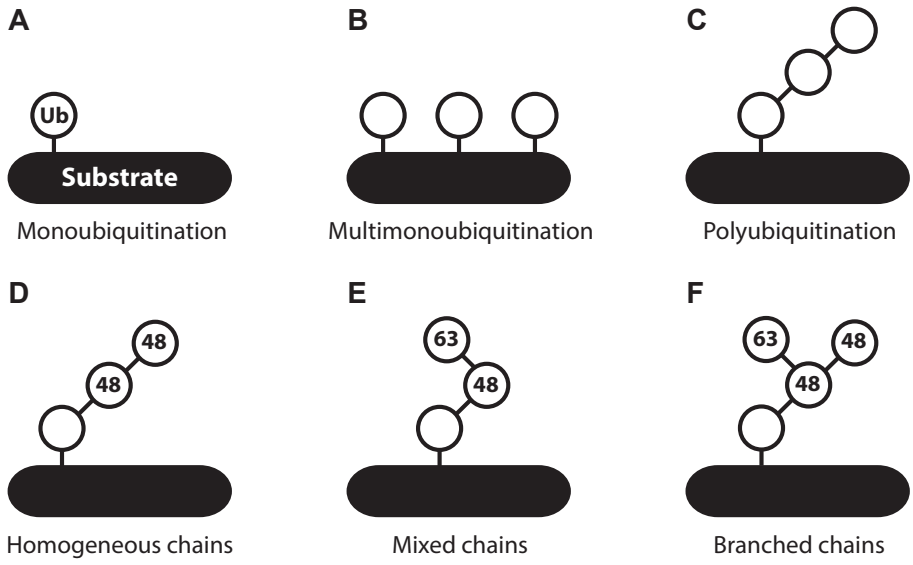

Figure I-2: Different forms of ubiquitination. Schematic representation of **(A)** monoubiquitination, **(B)** multimonoubiquitination, **(C)** polyubiquitination, **(D)** homogeneous polyubiquitin chains, **(E)** mixed polyubiquitin chains, and **(F)** branched polyubiquitin chains. Adapted from reference (69). **Abbreviations:** Ub, ubiquitin; 48/63, Lys48/63-linked.

molecules are attached to an equal number of lysine residues in a target protein (**Figure I-2B**). In the case of polyubiquitination, target proteins are decorated with chains consisting of two or more inter-linked ubiquitin molecules (**Figure I-2C**). Importantly, these polyubiquitin chains can take different forms as well, depending on the ubiquitin residue that is used to attach the C terminus of the adjoining ubiquitin molecule. Of the 76 amino acid residues that make up the ubiquitin molecule, eight can be involved in the formation of polyubiquitin chains. These residues are Met1 (resulting in linear polyubiquitin chains), Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, and Lys63, and all of these linkages have been detected in cells (70, 71). Interestingly, polyubiquitin chains of different linkage types have been shown to be structurally distinct, allowing for differential interactions (72-76). As a final means of variation, these polyubiquitin chains can be built up using only one linkage type (homogeneous chains; **Figure I-2D**), different linkage types in successive ubiquitin molecules (mixed chains; **Figure I-2E**), or even different linkage types in the same ubiquitin molecule (branched chains; **Figure I-2F**). Whereas the function of branched chains remains unknown, mixed chains have been found to play a role in signalling and protein trafficking (77-80).

The ubiquitin-like protein ISG15

A number of ubiquitin-like proteins have been described that share both structural and functional similarities with ubiquitin (for review, see (81)). In the context of this thesis, the interferon-stimulated gene-encoded protein of 15 kDa (ISG15) is of particular interest. This protein consists of two domains with a ubiquitin-like β-grasp fold and has the exact same C-terminal sequence motif as ubiquitin (Leu-Arg-Leu-Arg-Gly-Gly or LRLRGG). Like ubiquitin, ISG15 can be covalently attached to target proteins via its C terminus, a process which is referred to as ISGylation. Poly-ISG15 chains do not appear to be formed however. Functionally, ISG15 is considered to act as an antiviral molecule, although its exact mechanisms of action remain to be determined (for reviews, see (82, 83)). For example, unconjugated ISG15 has been suggested to act as an immunomodulatory cytokine (84), whereas ISGylation of newly translated viral proteins has been proposed to interfere with viral replication via steric hindrance of their interactions (85). In addition, ISGylation seems to be involved in the regulation of innate immune signalling factors and antiviral effector molecules (86, 87). Notably, the (ectopic) expression of ISG15, either free or conjugated, has been shown to inhibit replication of a wide variety of viruses (for a review, see (83)). In the case of arteriviruses, it has been shown that ectopic expression of conjugation-competent ISG15 (Gly-Gly C terminus), but not of conjugation-defective ISG15 (Ala-Ala C terminus), decreased replication of PRRSV in a cell culture-based assay (88).

Reversal of ubiquitination by deubiquitinases

One of the reasons why ubiquitination is such a useful regulatory posttranslational modification is the fact that it is readily reversible. Ubiquitin can be removed from target proteins by the action of deubiquitinases (DUBs) that cleave directly downstream of its C-terminal Gly-Gly motif, thus leaving no trace of its former ubiquitinated state on the target. Some DUBs are metalloproteases which use a Zn^{2+} ion for catalysis and possess a ubiquitin protease domain known as a JAB1/MPN/Mov34 (JAMM) domain. Most DUBs, however, are cysteine proteases and thus rely on a catalytic cysteine residue for proteolytic activity. These DUBs are grouped into four classes, based on the three-dimensional structure of their catalytic domains: the ubiquitin-specific proteases (USPs), the ovarian tumor proteases (OTUs), the ubiquitin C-terminal hydrolases (UCHs), and the Machado-Joseph disease proteases (MJDs) (for reviews, see (89, 90)). Even though all DUBs process the same C-terminal sequence motif of ubiquitin (LRLRGG), which is present in all ubiquitinated proteins, it appears that eukaryotic DUBs generally target a specific (set of) substrates (89, 90). This specificity might depend on several factors, including (the linkage type of) the ubiquitin or ubiquitin-like protein involved, the ubiquitinated target involved, protein localization, and the presence of additional binding partners. For example, a recent study shows that many OTU DUBs

display a distinct preference for one or two types of polyubiquitin chain linkage (91). In addition, it has been shown that the protease USP18 specifically targets the ubiquitin-like protein ISG15 (92). It is likely that the overall specificity of each DUB is determined by a combination of the factors mentioned above.

Ubiquitin and innate immunity

The innate immune response forms the first line of defence against invading pathogens and is, in addition to phosphorylation, extensively regulated through ubiquitination (for reviews, see (66, 68)). It relies on the recognition of pathogen-associated molecular patterns (PAMPs), such as viral proteins or nucleic acids, by cellular pattern recognition receptors (PRRs). Two main classes of PRRs exist in mammalian cells: the membrane-bound receptors, such as Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), and the cytosolic receptors, including the NOD-like receptors (NLRs), pyrin and HIN domain-containing (PYHIN) family members, the RIG-I-like receptors (RLRs), and a variety of newly described nucleic acid sensors such as cGAS (for a review, see (93)). Since the RLRs recognize cytosolic viral RNA (94-98), they are of particular importance for the recognition of invading RNA viruses (99, 100). The RLR family consists of three members called retinoic-acid inducible gene-encoded protein I (RIG-I), melanoma differentiation-associated protein 5 (MDA5), and laboratory of genetics and physiology protein 2 (LGP2). Although LGP2 was initially suggested to be a negative regulator of RLR-mediated signalling (101), later work suggests that it acts upstream of RIG-I/MDA5 and is in fact required for their activation, particularly in the case of picornavirus infection (102, 103). Upon recognition of viral RNA by RIG-I and MDA5, they bind to the adaptor molecule mitochondrial antiviral signalling protein (MAVS) (104). This molecule then forms large prion-like aggregates on the mitochondrial membrane (105), by which it initiates divergent signalling cascades that ultimately lead to the activation of the transcription factors interferon regulatory factor 3 (IRF3) and nuclear factor κB (NF-κB). These in turn induce the transcription of genes encoding type I interferons (IFNs) and other proinflammatory cytokines, which together are responsible for the induction of an antiviral state (for reviews, see (106, 107)). Upon its release from the cell, IFN binds to its cognate receptor through which it activates the JAK/STAT signalling pathway. In combination with IRF9, activated STAT1/2 transcription factors form a heterotrimeric complex called IFN-stimulated gene factor 3 (ISGF3). This complex is translocated to the nucleus where it binds to promoter regions encompassing IFN-stimulated response elements (ISREs) that direct the transcription of a myriad of IFN-stimulated genes including for example ISG15. **Figure I-3** provides a schematic overview of the RLR-mediated signalling pathways.

The innate immune signalling cascades rely on ubiquitin during many steps, two of which will be discussed here in more detail. Firstly, the activation of the PRR RIG-I is

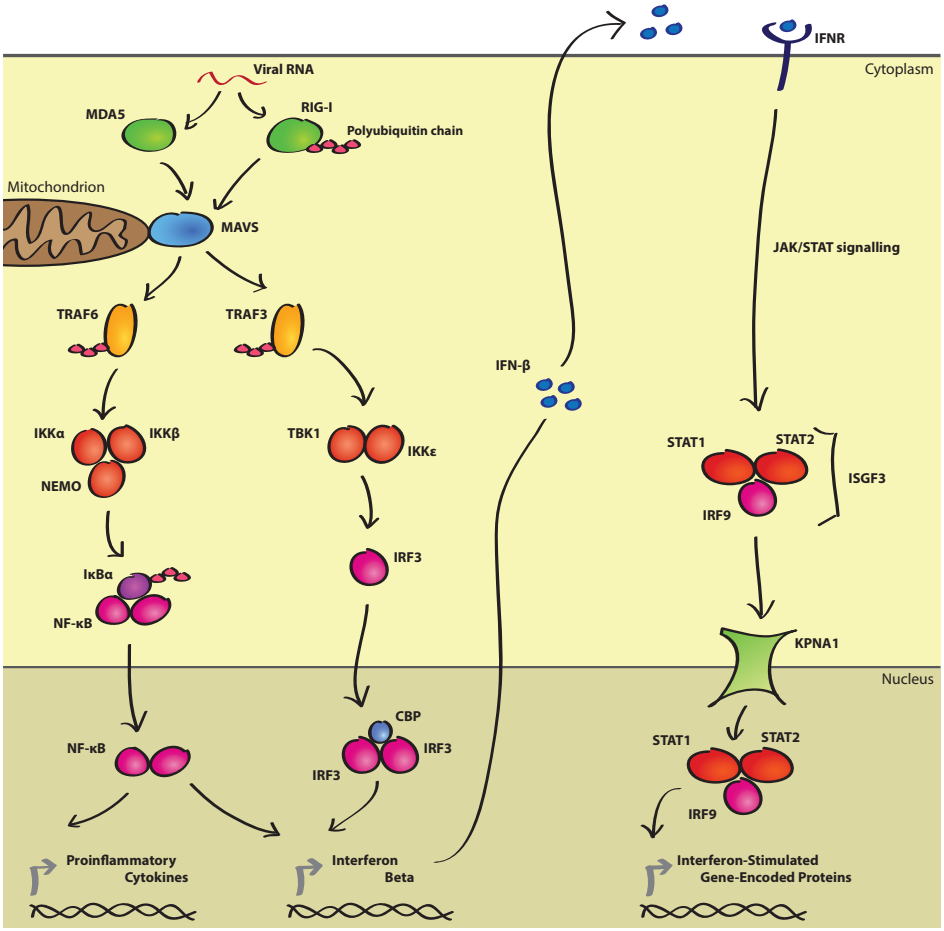

Figure I-3: Schematic overview of the RLR-mediated signalling pathways. Recognition of viral RNA by RIG-I and MDA5 leads to the activation of divergent signalling pathways that ultimately result in the transcription of genes encoding IFN-β and other proinflammatory cytokines. Following secretion, IFN-β binds to the IFN receptor and thereby activates the JAK-STAT signalling cascade that leads to the expression of interferon-stimulated genes. **Abbreviations:** CBP, CREB-binding protein; IFN-β, interferon beta; IFNR, interferon receptor; IKK, IκB kinase; IκBα, inhibitor of NF- κB; IRF, interferon regulatory factor; ISGF3, interferon-stimulated gene factor 3; JAK/STAT, janus kinase/ signal transducers and activators of transcription; KPNA1, karyopherin alpha 1; MAVS, mitochondrial antiviral signalling protein; MDA5, melanoma differentiation-associated protein 5; NEMO, NF-κB essential modulator; NF-κB, nuclear factor kappa B; RIG-I, retinoic acid-inducible gene-encoded protein I; RLR, RIG-I-lile receptors; STAT1/2, signal transducers and activators of transcription 1/2; TBK1, tank-binding kinase 1; TRAF3/6, TNF receptor-associated factor 3/6.

dependent on the presence of Lys63-linked polyubiquitin chains, although the exact nature of these chains remains debated. According to one view, recognition of viral RNA leads to the Lys63-linked ubiquitination of the C-terminal regulatory domain of RIG-I by the E3 ligase Riplet (108, 109). This induces a conformational change that exposes the two N-terminal caspase recruitment domains (CARDs) of RIG-I for Lys63-linked ubiquitination on Lys172 by the E3 ligase tripartite motif containing protein 25 (TRIM25) (110, 111). The latter ubiquitination event is thought to be essential for the interaction of RIG-I with MAVS and thus for the activation of downstream signalling. However, according to other studies, noncovalent binding of activated RIG-I (and MDA5) to unanchored short Lys63-linked polyubiquitin chains is responsible for an oligomerization-mediated interaction with MAVS and subsequent downstream signalling (112-114). Recent work suggests that it is likely a combination of covalent and noncovalent interactions with Lys63-linked polyubiquitin chains that results in full-blown activation of RIG-I (115). A second example of the role of ubiquitin in innate immunity lies in the activation of the transcription factor NF-κB. This protein is normally retained in the cytoplasm by the inhibitor of NF-κB protein alpha (IκBα). However, activation of innate immune signalling cascades leads to the Lys48-linked ubiquitination of IκBα, which is subsequently degraded by the proteasome (116). NF-κB is thus released from the inhibitory complex and free to translocate to the nucleus where it can induce the transcription of its target genes. Although there are many more examples of ubiquitination in innate immune signalling, these two nicely underline how innate immune signalling cascades can be influenced by both degradative and non-degradative ubiquitination.

Since the inappropriate activation, either in timing or magnitude, of innate immune signalling pathways can have disastrous effects, many mechanisms are in place to prevent this from happening. One of these is the presence of cellular DUBs that are involved in downregulating innate immune activation. For example, the ubiquitin-specific protease cylindromatosis (CYLD) has been shown to deubiquitinate RIG-I and various other innate immune signalling factors, thus preventing downstream signalling (117, 118). In addition, the OTU proteases otubain 1 and 2 (OTUB1/2) have been shown to deubiquitinate and thus inactivate the innate immune adaptor proteins TNF receptor-associated factor (TRAF) 3 and 6 (119). These are only two examples of how cellular DUBs are employed to keep innate immune signalling from running out of bounds, but many more have been documented (68, 118).

NIDOVIRUS DEUBIQUITINASES

Considering the importance of ubiquitination in cellular homeostasis in general and the activation of innate immune signalling in particular, it is perhaps not surprising that many viruses encode proteins that interact with the ubiquitin system (for reviews, see (66, 120)). Indeed it was shown that several of the accessory protease domains encoded by arteri- and coronaviruses act as DUBs in addition to mediating specific steps in the proteolytic maturation of the replicase polyproteins (121-130). For arteriviruses, the first indication that PLP2 might possess DUB activity came from a bioinformatics analysis that revealed a remote sequence similarity between this enzyme and members of the OTU superfamily of proteases (131). Experimental support for this hypothesis later came from Frias-Staheli *et al.*, who showed that ectopic expression of either EAV or PRRSV nsp2, which encompasses the PLP2 domain, results in an overall decrease in cellular ubiquitination (121). In the case of the coronaviruses, the DUB activity of SARS-CoV PL2pro was initially predicted based on structural models that revealed similarity with DUBs of the USP class (132) and this hypothesis was subsequently confirmed using *in vitro* cleavage assays (123, 124). Following this discovery, DUB activity was also confirmed for PL2pro of human coronavirus NL63 (125), mouse hepatitis virus (MHV) (126), porcine epidemic diarrhea virus (PEDV) (128), and MERS-CoV (127, 129), and PL1pro of transmissible gastroenteritis virus (TGEV) (122). Since ubiquitin is such an important player in innate immune regulation, many studies have proposed a role for arteri- and coronavirus DUBs in innate immune evasion (88, 121, 126-130, 133-137). However, since all of these enzymes (PLP2, PL1pro, and PL2pro) are also essential for viral replicase maturation and can therefore not be easily mutated without affecting virus viability, it has been impossible to show that their DUB activity is important for the evasion of innate immune signalling in the context of a nidovirus infection.

OUTLINE OF THIS THESIS

The work described in this thesis provides novel insights into the structural features and function of arterivirus PLP2 and into the role of its DUB activity in the evasion of ubiquitin-regulated innate immune signalling. The experiments described in *Chapter 2* show that PLP2 DUB activity is conserved among EAV, PRRSV-I, PRRSV-II, LDV, and SHFV. In addition, it is shown that arterivirus PLP2 can decrease ubiquitination of RIG-I upon ectopic expression, suggesting that this is one of the mechanisms by which arteriviruses evade innate immunity. In *Chapter 3* a fluorescence polarization-based *in vitro* small-molecule inhibitor screen is described, which has resulted in the

identification of several inhibitors of PLP2 activity that limit EAV replication in cell culture. C*hapter 4* presents the crystal structure of the EAV PLP2 domain in complex with ubiquitin, which forms the basis for the design of PLP2 mutants that are defective in DUB activity but have retained the ability to process the nsp2|3 junction in the viral replicase polyproteins. These mutants were subsequently employed to show that PLP2 DUB activity is indeed important for the inhibition of innate immune signalling during EAV infection of primary equine cells. *Chapter 5* provides the results of a small-scale vaccination trial in horses where the level of protection provided by a PLP2 DUB-negative vaccine virus was compared with its DUB-competent parental strain. Although the PLP2-mutant virus appeared to provide slightly better protection against challenge than its parental counterpart, no firm conclusions could be drawn from the data. *Chapter 6* contains a structural and functional comparison of OTU DUBs encoded by widely different RNA virus groups (arteri-, nairo-, and tymoviruses). Finally, *Chapter 7* encompasses a general discussion that places the novel findings described in this thesis in the framework of existing knowledge on arterivirus proteases and immune evasion strategies and in which some future research directions concerning the target specificity and putative deISGylating activity of arterivirus PLP2 are delineated.

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