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A DEUBIQUITINASE THAT COUNTERACTS INNATE IMMUNITY

As is the case for many proteases encoded by mammalian +RNA viruses, the arterivirus proteases play a crucial role in the viral replication cycle. Together, they are responsible for sculpting the replicase polyproteins into active replication and transcription complexes and without their activity viral infection would quickly come to a grinding halt. Consequently, identification and design of specific protease inhibitors form promising approaches in the search for antiviral drugs, which can greatly benefit from the in-depth characterization of the structural and functional aspects of viral proteases. In addition to playing a direct role in replication, one of the arterivirus protease domains (papain-like protease 2; PLP2) has previously been suggested to be involved in the evasion of innate immune activation by means of its putative deubiquitinase (DUB) activity (1). Since the innate immune response is presumed to be pivotal for eliciting a timely and adequate adaptive immune response, increased knowledge of viral innate immune evasion strategies will likely make an important contribution to the future design of improved vaccines.

The work described in this thesis provides novel insights into the structural features and (multi)functionality of arterivirus PLP2. Not only have we clearly demonstrated the in vitro DUB activity of this enzyme and the conservation of this activity across the arterivirus family (Chapter 2), we have also shown how this DUB activity can be exploited in the search for antiviral compounds (Chapter 3). Furthermore, the crystal structure of equine arteritis virus (EAV) PLP2 answered some long-standing questions regarding the architecture of this domain and allowed us to demonstrate the importance of its DUB activity in counteracting the innate immune response (Chapter 4). By means of targeted mutagenesis, we have succeeded in separating the polyprotein processing and DUB functions of PLP2, which finally allowed for a direct assessment of the role of this enzyme in innate immune evasion during infection. Following up on this work, an in vivo experiment was performed to evaluate the potential use of PLP2 DUB-negative viruses in arterivirus vaccine design (Chapter 5). Below, this newly obtained information will first be discussed in the context of the existing framework of knowledge regarding arterivirus proteases in general and PLP2 in particular. Next, I will discuss our current understanding of arterivirus innate immune evasion strategies and the potential use of this knowledge in the design of novel arterivirus vaccines. Some future research directions will then be delineated, in particular concerning the target specificity and putative deISGylating activity of arterivirus PLP2. This general discussion will be concluded with some words on the multifunctional nature of proteases encoded by diverse +RNA virus groups.
Characterization of arterivirus proteases

The identification of arterivirus protease domains commenced with the elucidation of the EAV genome sequence in 1991, which revealed the presence of a chymotrypsin-like serine protease (SP) consensus sequence in the subunit that would later be named nsp4 (2). Further studies confirmed the SP to be the main arterivirus protease, which is responsible for processing of the majority of ORF1a-encoded cleavage sites and all ORF1b-encoded cleavage sites in the replicase polyproteins (pp1a and pp1ab) (3, 4). In addition, initial sequence analysis of the EAV polyproteins revealed some similarity with cellular papain-like proteases in the C-terminal region of the protein now known as nsp1 (2), and later studies confirmed the activity of this domain towards the nsp1|nsp2 cleavage site (5, 6). Because of the subsequent identification in other arteriviruses of one or two additional upstream papain-like protease domains (7, 8), the nsp1 protease domains are now referred to as papain-like protease 1α (PLP1α), PLP1β, and PLP1γ. In porcine reproductive and respiratory syndrome virus (PRRSV) and lactate dehydrogenase-elevating virus (LDV), which both contain two active nsp1 proteases, PLP1α and PLP1β mediate the release of two nsp1 subunits, nsp1α and nsp1β. In the case of EAV, a presumed substitution of the PLP1α catalytic cysteine has rendered this domain inactive (7), resulting in the release of a single nsp1 subunit encompassing only one active protease domain, i.e. PLP1β. In the case of simian hemorrhagic fever virus (SHFV), a combination of sequence analysis and recent functional studies has revealed an even more complicated organisation of the nsp1 region, encompassing three active papain-like protease domains (9).

Finally, the analysis of EAV pp1a processing revealed the involvement of a third (viral or cellular) protease that was responsible for cleavage of the nsp2|nsp3 site (5). An extensive search involving both bioinformatics analysis and mutagenesis eventually lead to the identification in the N-terminal region of nsp2 of a cysteine protease (CP) with both papain-like and chymotrypsin-like amino acid sequence characteristics (10). The subsequent discovery of a cellular papain-like protease with similar sequence characteristics (11), prompted the eventual renaming of this CP domain into papain-like protease 2 (PLP2). Later, protease activity was also experimentally confirmed for the PLP2 domain of PRRSV (12). Presumably, all arterivirus proteases have now been identified, although the possibility of future additions cannot be excluded.

The road to structural clarification

The three-dimensional structures of the arterivirus proteases were resolved in the same order in which they were initially described. The first was that of the EAV SP (Figure VII-1A and (13)), followed some years later by the elucidation of the PRRSV SP
Figure VII-1: Overview of nidovirus protease crystal structures. Crystal structures of (A) EAV nsp4 (PDB ID: 1MBM) (13), (B) SARS-CoV nsp5 (PDB ID: 1UJ1) (15), (C) PRRSV nsp1α (PDB ID: 3IFU) (16), (D) PRRSV nsp1β (PDB ID: 3MTV) (17), (E) EAV PLP2 (PDB ID: 4IUM) (Chapter 4), (F) transmissible gastroenteritis virus (TGEV) PL1pro (PDB ID: 3MP2) (21), and (G) SARS-CoV PL2pro (PDB ID: 2FE8) (29). Chymotrypsin-like main proteases are depicted in green and papain-like accessory proteases are depicted in blue. Active site residues are shown in red as sticks. EAV PLP2 was crystallized in complex with ubiquitin, which is not shown here for clarity. Abbreviations: CTD, C-terminal domain; CTE, C-terminal extension; NTD, N-terminal domain; UBL, ubiquitin-like domain; ZF, zinc finger; ZFD, zinc finger domain.
crystal structure (14). As predicted (4), the arterivirus SP adopts the two β-barrel fold that is characteristic of chymotrypsin-like proteases. In this respect, it resembles the main proteases found in the distantly related subfamily Coronavirinae (Figure VII-1B and (15)), with the exception that the latter exploit a cysteine instead of a serine as the nucleophilic residue that drives catalysis. Next, the crystal structures of the PRRSV nsp1α and nsp1β subunits (encompassing the PLP1α and PLP1β domains) became available (Figure VII-1C and D and (16, 17)), which both display the predicted papain-like α+β fold.

With the addition of the EAV PLP2 domain, the collection of arterivirus protease structures is now nearing completion, with only PLP1γ lacking (Figure VII-1E and Chapter 4). The structure of PLP2 remained elusive for a long time due to difficulties with its purification, but these were solved, in part, by switching to the use of a covalent protease-substrate complex that stabilized the protein. The PLP2 domain indeed also proved to adopt the α+β fold that is characteristic of papain-like proteases, but in addition displayed similarity with enzymes belonging to the ovarian tumor (OTU) superfamily of proteases, as had previously been suggested (18). The functional implications of this resemblance will be addressed below. Furthermore, the three-dimensional structure of EAV PLP2 confirmed the previous suggestion of a nucleophilic cysteine (Cys270) and histidine (His332) being responsible for catalysis (10), and revealed that an additional residue (Asn263) completes the catalytic triad (Figure IV-1D). Finally, the PLP2 crystal structure also confirmed the presence of a previously predicted zinc finger (10), which seems to be involved in protease-substrate interaction (Figure IV-2A). The incorporation of a zinc finger motif within the OTU-fold appears to be unique for arterivirus PLP2, which is why we have proposed that this enzyme prototypes a novel subclass of zinc-dependent OTU proteases (Chapter 4).

**Arterivirus PLP2 is an OTU deubiquitinase**

Several years after its identification in 1995, an extensive bioinformatics analysis revealed that the arterivirus PLP2 domain shares remote sequence similarity with enzymes belonging to the OTU superfamily of proteases (18), which consists of papain-like cysteine proteases that often display DUB activity (19). Subsequent work by Frias-Staheli et al. (2007) showed that ectopic expression of EAV and PRRSV nsp2 resulted in an overall decrease of the level of ubiquitinated proteins, which was consistent with the hypothesis that the PLP2 domain functions as a DUB (1). In Chapter 2 of this thesis we provided the first in vitro evidence that purified recombinant EAV PLP2 exhibits DUB activity (Figure II-1). Moreover, our in vitro results uncovered a slight preference of EAV PLP2 for Lys63- over Lys48-linked polyubiquitin chains. These findings are in line with a recent report in which it is shown that also PRRSV PLP2 displays an in vitro preference for Lys63-linked di-ubiquitin, followed by (in order of decreasing
preference) Lys11-, Lys29-, Lys6-, Lys27-, and Lys48-linked di-ubiquitin (20). Activity towards linear chains could not be detected. Whether EAV PLP2 displays a similar pattern of linkage preferences, and to what extent these in vitro results correspond to the natural situation, remains to be investigated. In addition to firmly establishing the DUB activity of PLP2, we showed that this property is very likely conserved among all four currently recognized arterivirus species (Figure II-3). This study also provided the first (preliminary) experimental evidence of proteolytic activity for the LDV and SHFV PLP2 domains.

Importantly, the finding that arterivirus PLP2 functions as a DUB had implications for the delineation of the nsp2|nsp3 cleavage site. Based on estimated nsp2 and nsp3 sizes, the idea that papain-like proteases often cleave between two small amino acid residues, and a mutagenesis experiment showing that a Gly831Pro mutation hampered cleavage, it had previously been suggested that EAV PLP2 cleaves between pp1a residues Gly831 and Gly832 (4). According to this scenario, the nsp2|nsp3 cleavage site would be $a_{827}$FRLIG|GW$_{833}$. However, the fact that DUBs generally cleave directly downstream of a C-terminal LRLRG sequence, now made it much more likely that cleavage actually occurs downstream rather than upstream of residue Gly832, thus changing the cleavage site to $a_{827}$FRLIGG|W$_{833}$. This idea was further supported by the crystal structure of PLP2 in complex with ubiquitin, which showed that the two C-terminal glycine residues of ubiquitin (the second having been replaced with a reactive group) fit nicely into the groove leading up to the active site (Chapter 4). Given the similarity between the C termini of ubiquitin and EAV nsp2, it seems likely that the latter is also cleaved downstream of the double glycine motif. Nevertheless, definitive confirmation of this hypothesis still requires additional experimental work, such as sequencing of viral cleavage products.

Interestingly, arteriviruses are not the only viruses that harbour DUB activity. For example, also the distantly related coronaviruses encode papain-like proteases with DUB activity (21-28). In contrast to the arterivirus OTU DUBs, these coronavirus enzymes belong to the ubiquitin-specific protease (USP) class of DUBs (Figure VII-1F and G and (21, 29)). Other viral proteases suggested to act as DUBs are the USP-like leader protease of the picornavirus foot-and-mouth disease virus (FMDV) (30) and the OTU-like protease domain encoded by turnip yellow mosaic virus (TYMV) (31), which as the name indicates is a plant virus. Although these are all examples of DUBs encoded by +RNA viruses, their presence is certainly not restricted to this particular group of viruses. Also the negative-strand RNA viruses belonging to the nairovirus genus encode an OTU DUB in the N-terminal region of their polymerase protein (1, 32-34) and DUBs are expressed by a number of DNA viruses, including several herpesviruses (35-39) and an adenovirus (40). Since bioinformatics-based analyses have been instrumental in the discovery of DUB activity for many of these viral proteases (18, 30, 41), the
increased analytical power of such techniques in combination with the increasing number of available genome sequences might lead to the future identification of even more virus-encoded DUBs.

**Deubiquitinase-based screens for antiviral compounds**

In addition to being an interesting feature of arterivirus PLP2 in itself, the DUB activity of this and other +RNA viral enzymes opens up novel possibilities for the search for antiviral protease inhibitors since it can be used as a read-out for proteolytic activity. Because of their essential role in replicase maturation, +RNA viral proteases form a promising target for the design of antiviral drugs. This is exemplified by the recent FDA approval for clinical use of two compounds that inhibit the main protease of hepatitis C virus and the use of several protease inhibitors for the treatment of HIV infection (for reviews, see (42, 43)). However, the search for such compounds often depends on time-consuming cell culture-based infection assays and would therefore likely benefit from the design of (high-throughput) *in vitro* screens that allow for initial selection of promising compounds without the safety constraints imposed by working with live viruses. One of the advantages of using a DUB-based screening approach is that a similar experimental set-up can be used to identify inhibitors of proteases encoded by different viruses, without the need to design specific substrates for each individual protease.

Using EAV PLP2 as a model, we showed that a recently developed *in vitro* DUB assay based on fluorescence polarization (44-47) can be applied for high-throughput *in vitro* screens to identify inhibitors that decrease viral replication (*Chapter 3*). This assay depends on a ubiquitin substrate that is covalently linked via an isopeptide bond to a rhodamine-based fluorophore (TAMRA) and on a decrease in polarization of the emitted light that is used as a read-out for substrate cleavage. Because of the ease of production of the substrate and the low reaction volumes required, this DUB assay is particularly well suited for use in high-throughput experimental set-ups. Using this assay, we have screened a library of 335 suspected DUB inhibitors for activity against EAV PLP2. Of the five compounds that were found to inhibit PLP2 DUB activity (*Figure III-1C*), four also inhibited replication of a GFP-expressing reporter virus to a reasonable extent (*Figure III-2*). Although the demand for antiviral compounds targeting viruses of livestock and other, especially non-domesticated, animals is extremely limited, these results do show that this DUB-based *in vitro* screen can, in principle, be applied for the identification of compounds that inhibit replication of DUB-encoding viruses. It would therefore be interesting to use this set-up to screen for compounds that inhibit the DUBs encoded by for example the SARS- and MERS-coronaviruses, for which the search for antiviral compounds is still ongoing (for a recent review, see (48)).
Evasion of Innate Immunity by Arteriviruses

PLP2 inhibits innate immune activation

As described above, the PLP2 domain was one of the last arterivirus proteases both to be identified and to be crystallized. In contrast, this enzyme was actually the first arterivirus protein domain suggested to be involved in the evasion of innate immune activation. It had long been established, in particular for PRRSV, that arteriviruses induce only very low levels of type I interferons (IFNs) (49-51), but the first viral proteins and mechanisms responsible for counteracting the IFN response were not identified until years later. The extensive ubiquitin-mediated regulation of innate immunity first prompted Frias-Staheli et al. (2007) to suggest a possible role for arterivirus PLP2 in innate immune evasion (1). This hypothesis was supported by their finding that ectopic expression of arterivirus nsp2 inhibited TNFα-mediated activation of NF-κB, an important transcription factor for innate immune signalling. Subsequent work, performed in several laboratories including our own, provided further support for a role of arterivirus PLP2 in innate immune evasion ((52-54) and Chapter 2). For example, we showed that ectopic expression of nsp2 or PLP2 inhibited RIG-I-mediated activation of the IFNβ-promoter in a luciferase-based reporter assay (Figure II-5) and in addition decreased the level of RIG-I ubiquitination (Figure II-7). Nevertheless, definitive proof for such a role during infection remained elusive mainly because of the primary role of arterivirus PLP2 in replicase maturation, which precluded straightforward inactivation of this domain.

To overcome this problem, we initially attempted to decouple the proteolytic activity of the PLP2 domain from processing of the nsp2|nsp3 junction by replacing the latter with a “self-cleaving” peptide sequence from the 2A protein of FMDV. This sequence has been shown to induce a ribosomal skipping event that prevents the formation of a particular peptide bond, thereby resulting in the co-translational formation of two discrete protein products from one open reading frame (55, 56). The insertion of the FMDV 2A sequence in an nsp2-nsp3 expression construct resulted in the near-complete and PLP2 activity-independent separation of nsp2 and nsp3 upon transfection in eukaryotic cells (unpublished data). However, recombinant EAV encoding this sequence turned out to be non-viable, preventing further analysis.

In Chapter 4, we described how the elucidation of the crystal structure of EAV PLP2 allowed for the separation of its polyprotein processing and DUB functions by site-directed mutagenesis of the ubiquitin interaction site (Figure IV-3), which opened up the possibility of studying the role of PLP2 DUB activity independently of its role in replicase maturation. Using this approach, we could show that virus mutants lacking PLP2 DUB activity showed similar replication kinetics as wild-type virus (Figure IV-5A
to C), yet induced approximately 8-fold higher levels of interferon beta (IFNβ) mRNA upon infection of primary equine cells (Figure IV-6). We were thus able to show for the first time that the DUB activity of arterivirus PLP2 is indeed important for the evasion of innate immune activation during infection. Notably, the replication kinetics of the above-mentioned mutants was tested in a single-cycle infection experiment and was likely for this reason not affected by the observed enhanced innate immune activation. However, these mutants did show an altered plaque phenotype in primary equine cells, with plaques of the mutant viruses being more diffuse than those induced by the wild-type virus (unpublished data, Figure VII-2). This observation suggested that viruses lacking PLP2 DUB activity induce a less pronounced cytopathic effect, which is likely due to the enhanced activation of innate immunity. In addition to an effect of PLP2 DUB-mutations on the expression of IFNβ mRNA, we also observed a significantly enhanced expression of the interferon-stimulated gene MX1 (Figure IV-6B). Whether this is due to a secondary effect of increased IFNβ production, or whether PLP2 can also inhibit downstream interferon-mediated signalling remains to be investigated.

Interestingly, DUB-mediated evasion of innate immunity appears to be a strategy that is commonly used by diverse virus groups. Although not yet confirmed in infected cells, several coronaviruses have been suggested to counteract innate immune activation by means of the DUB activity of their papain-like protease domains (25-28, 57-61). In addition, ectopic expression of the FMDV leader protease was shown to decrease Sendai virus-mediated activation of the IFNβ-promoter (30). Finally, also the OTU DUBs encoded by nairoviruses (1, 62, 63) and some herpesvirus-encoded DUBs
have been suggested to be responsible for innate immune evasion by these viruses.

**Teaming up against the host**

In addition to nsp2, several other arterivirus proteins have been suggested to be involved in the evasion of innate immune responses. For example, an early study by Beura et al. showed that individual expression of PRRSV nsp1α-β, nsp4, or nsp11 inhibits activation of the IFNβ-promoter by the transcription factor IRF3 (66). Following up on this work, research efforts have mainly focused on elucidating the roles of PRRSV nsp1α and nsp1β in innate immune evasion (8, 66-74). Although both of these proteins encompass protease domains, their immune evasive activities do not appear to rely on proteolytic activity per se (67, 75). This is consistent with the notion that arterivirus PLP1 domains cleave only in cis, as was found for EAV PLP1β (6). Structural analysis showed that, following cleavage, the C-terminal tails of nsp1α and -β remain in the catalytic cleft, obstructing further proteolytic activity (16, 17). In the case of PRRSV nsp1α, innate immune evasion was shown to depend on the induction of proteasomal degradation of CREB-binding protein (CBP) (67, 71), which normally interacts with dimerized IRF3 upon translocation into the nucleus and is essential for its transcriptional activity. The nsp1α-mediated degradation of CBP thus prevents IRF3-mediated activation of the IFNβ-promoter. Mutational analysis furthermore showed that the activity of nsp1α towards CBP likely depends on its zinc finger domain (67, 68).

Nsp1β has been shown to inhibit both activation of the IFNβ-promoter and IFN-induced JAK-STAT signalling (8, 66, 76). Whereas the exact mechanism of inhibition of IFNβ-promoter activation remains unclear, the inhibition of JAK-STAT signalling was reported to depend on nsp1β-mediated degradation of karyopherin-α1 (KPNA1) (73). This nuclear pore protein is responsible for the IFN-induced nuclear import of IFN-stimulated gene factor 3 (ISGF3), which consists of a heterotrimeric complex including the transcription factors STAT1, STAT2, and IRF9. Once transported into the nucleus, ISGF3 binds to promoter regions encompassing IFN-stimulated response elements (ISREs) to induce the transcription of IFN-stimulated genes (ISGs). The nsp1β-mediated degradation of KPNA1 thus prevents the expression of ISGs and thereby the induction of an antiviral state. To date, three reports have been published showing that viruses harboring certain mutations in PRRSV nsp1α or nsp1β induce higher expression levels of genes involved in innate immunity than wild-type virus upon infection in cell culture or in vivo, although these observations were not directly linked to a particular activity or target of these proteins (72, 75, 77).

Besides the above-mentioned preliminary work by Beura et al. (66), the effect of nsp4 on innate immune signalling has not been thoroughly investigated. Neverthe-
less, it was recently reported that the PRRSV main protease specifically cleaves the innate immune signalling factor NEMO, thereby preventing its activation (78). This finding nicely adds to the already considerable number of +RNA virus-encoded main proteases that have previously been shown or suggested to inhibit innate immune signalling pathways (79-90). Regarding PRRSV nsp11, there is one study that shows that, in a luciferase-based reporter assay, ectopic expression of this protein inhibits activation of the IFNβ-promoter upon stimulation with the dsRNA analogue poly(I:C), an effect suggested to depend on the endoribonuclease activity of the protein (91). However, because of this activity, ectopic expression of nsp11 has previously been suggested to be extremely toxic to eukaryotic cells (92) and the obtained results concerning a direct role in innate immune evasion for this enzyme should therefore be interpreted with extreme caution. Finally, ectopic expression of the nucleocapsid (N) protein of PRRSV has been shown to inhibit the expression of IFNβ and ISG15 mRNA upon stimulation with poly(I:C) (93). For both nsp11 and N, elucidation of their exact mechanisms of action and an assessment of the importance of their putative immune evasive activities during infection await further experimental clarification. Figure VII-3 provides a schematic overview of the current status of knowledge concerning the evasion of innate immunity mediated by arterivirus proteins.

In addition to actively interfering with innate immune signalling, arteriviruses appear to have evolved mechanisms to prevent the initial recognition of their RNA by cellular sensors. For example, arteriviruses are presumed to have 5’-capped genomes (94), which is an effective means of hiding the phosphorylated 5’ end of an RNA from recognition by the pattern-recognition receptor RIG-I. Another feature of arterivirus replication is the formation of extensive intracellular membrane modifications, consisting predominantly of double-membrane vesicles (DMVs). The interior of these DMVs has been shown to contain (presumably viral) double-stranded RNA and does not appear to be connected to the cytosol (95). For this reason, one of the hypotheses concerning DMV formation is that they are employed to shield viral RNA from pattern-recognition receptors, thereby preventing innate immune activation.

**Increasing interferon induction to improve arterivirus vaccines**

It has been suggested that the ability of arteriviruses to evade innate immune activation is one of the reasons why vaccines do not provide optimal protection against infection with these viruses (96). Especially in the case of PRRSV, the virus appears to induce suboptimal levels of IFNs, which ultimately results in late and very limited neutralizing antibody and cell-mediated (i.e. adaptive) immune responses. In support of this idea, recent work has shown that infection with a particular PRRSV strain (A2MC2) that induces higher levels of IFN than other strains (including a MLV strain), triggers an earlier and more potent induction of neutralizing antibodies compared to strains that
Figure VII-3. Overview of innate immune evasion mediated by arterivirus nonstructural proteins. Arteriviruses have been suggested to counteract innate immune signalling pathways in a variety of ways. Firstly, \textit{PRRSV nsp1α} was found to induce the proteasomal degradation of CBP, and \textit{PRRSV nsp1β} was shown to mediate the degradation of KPNA1. In addition, \textit{PRRSV nsp4} was recently found to cleave the innate immune signalling factor NEMO. Finally, the \textit{arterivirus PLP2} domain has been shown to decrease the ubiquitination of RIG-I in an ectopic expression system and of \textit{IkBa} \textit{in vitro}. \textbf{Abbreviations:} CBP, CREB-binding protein; IFN-β, interferon beta; IFNR, interferon receptor; IKK, IkB kinase; IkBa, 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 1; RLR, RIG-I-like receptors; STAT1/2, signal transducers and activators of transcription 1/2; TBK1, tank-binding kinase 1; TRAF3/6, TNF receptor-associated factor 3/6.
induce lower IFN levels (97). Moreover, the neutralizing antibodies induced by the A2MC2 strain even proved to possess in vitro cross-reactivity towards a heterologous strain (97). Whereas for arteriviruses the available data on this subject is limited, a considerable body of work is available on the potential of increasing IFN induction in the context of the rational design of improved influenza vaccines. Specifically, non-structural protein 1 (NS1) of this virus has been found to possess pleiotropic immune evasive capabilities and deletion or mutation of this protein results in an enhanced induction of type I IFNs (for a review, see (98)). Interestingly, a large number of studies (including several in vivo trials) support the idea that mutating the influenza NS1 protein provides a promising strategy for the design of live attenuated vaccines (for a review, see (99)), which might also be applied to other virus groups that evade the host innate immune response.

Once we had established that arterivirus PLP2 DUB activity is important for the evasion of innate immunity during infection (Chapter 4), we hypothesized that a vaccine virus lacking PLP2 DUB activity provides better protection against subsequent challenge than its DUB-competent counterpart. To test this hypothesis, we performed an in vivo trial in which we vaccinated horses with PLP2 DUB-negative or DUB-competent cell culture-adapted EAV and challenged them with moderately virulent EAV KY84 (Chapter 5). The results of this trial show that PLP2 DUB-negative and DUB-competent EAV induce similar neutralizing antibody titers which do not increase upon challenge (Figure V-2B). In addition, when used as a vaccine both viruses were shown to induce similar levels of ISG15 and MX1 mRNA (Figure V-3C and D). This result suggests that there are no (detectable) differences in the activation of innate immunity between these two viruses, but assessment of additional targets is needed to confirm this. Furthermore, we could show that PLP2 DUB-negative EAV replicates in vivo, although slightly less efficiently than its DUB-competent counterpart (Figure V-3A). Finally, it turned out that the DUB-competent vaccine virus already provided such high levels of protection in our experimental set-up, that we could barely detect viral RNA in the blood of vaccinated horses after challenge. This unfortunately made it very difficult to assess differences between the two viruses, although close examination of the data did suggest that we could detect low levels of viral RNA more often in horses that had received the DUB-competent vaccine virus than in horses that had received the PLP2 DUB-negative virus (Figure V-3B).

Taken together, these results support the idea that a vaccine virus lacking PLP2 DUB activity can provide better protection against a challenge infection than its DUB-competent counterpart, but additional experiments are definitely required. In hindsight, we should perhaps have performed a more stringent challenge, for example by using a higher dose of challenge virus. However, the best option will likely be to perform a similar trial based on a parental virus that itself is a less efficacious
vaccine, preferably one of the PRRSV modified live virus (MLV) vaccines. To this end, it will be essential to first design a viable mutant PRRSV that lacks PLP2 DUB activity. Furthermore, our results have shown that knocking out PLP2 DUB activity has only a minimal effect on virus replication. This opens up the possibility of combining PLP2 mutations with mutations in other immune evasive domains, e.g. nsp1α or nsp1β, in order to potentially increase the immunogenicity of the vaccine virus in a synergistic manner. As a point of caution however, it needs to be noted that increasing innate immune activation too strongly can have unwanted effects. Not only can this result in over-attenuation leading to a lack of replication that is not beneficial to the induction of protective adaptive immunity, it can also result in increased pathogenesis due to immunopathology, as was previously reported for a certain PRRSV strain (100). The effects of reducing the immune evasive capacity of a vaccine candidate should thus be carefully monitored and a delicate balance is likely to be required. In conclusion, the results obtained by us and others definitely warrant further investigations into the potential of targeting (PLP2) DUB and other immune evasive activities to increase the efficacy of (arterivirus) vaccines.

IN SEARCH OF THE (SPECIFIC) TARGET(S) OF ARTERIVIRUS PLP2

The interaction of PLP2 with the viral polyprotein

The crystal structure of EAV PLP2 in complex with ubiquitin nicely defined the interaction surface between these two proteins (Chapter 4). Furthermore, mutations in this area were shown to specifically affect only the DUB activity of PLP2, which suggested that the interaction with the viral polyprotein relies on an alternative interaction surface of the protease. However, the exact location of the latter remains to be determined, just like the exact sequences flanking the nsp2|nsp3 cleavage site that are required for this interaction. Considering that PLP2 appears to use alternate surfaces for its interaction with ubiquitin and the viral polyprotein, it seems unlikely that the C-terminal domain of nsp2 adopts a ubiquitin-like fold to support the interaction with the PLP2 domain. Comparative sequence analysis does reveal a cluster of conserved cysteine residues in the C-terminal region of nsp2, which might be part of a structural element that is involved in the interaction with PLP2. Crystallization of a (covalent) complex consisting of PLP2 and the C-terminal region of nsp2 might shed more light on the interaction between the two. However, to date our preliminary attempts to obtain such a complex have been unsuccessful due to the instability of an expression product representing the C-terminal domain of nsp2. In the future, this problem might be solved by optimization of the precise region incorporated in this expression construct. An enhanced understanding of the interaction of PLP2 with its viral substrate
might reveal additional options for the separation of DUB and polyprotein processing activities.

The promiscuous nature of arterivirus deubiquitinases

One of the most striking and perhaps puzzling features of arterivirus PLP2 DUB activity is its apparent promiscuity. From the ectopic expression experiments described in literature and in this thesis it is evident that this enzyme can have a dramatic and seemingly nonspecific effect on the levels of ubiquitination of cellular proteins (see for example Figure II-3B and C and (1, 53)). This promiscuity is not unique for arterivirus PLP2, since it has also been demonstrated for coronavirus papain-like proteases upon ectopic expression of SARS-CoV, MERS-CoV, HCoV-NL63, and PEDV PL2pro (26, 28, 60). Under these experimental conditions, one might assume that the observed general effect on ubiquitination is merely an artefact of ectopic expression. Indeed, the respective protease domains were removed from their natural context, in which they are part of a much larger multi-domain nonstructural protein that resides in a multi-subunit replication and transcription complex and localizes to an intricate network of membrane modifications, all of which may influence their substrate access. However, although somewhat less pronounced, also in infected cells arterivirus PLP2 appears to have a strikingly general effect on the endogenous ubiquitination levels of cellular proteins, which correlates with the functionality of its DUB activity (Figure IV-5D). Moreover, a similarly general effect on ubiquitination levels has been reported in cells expressing epitope-tagged ubiquitin that are subsequently infected with SARS-CoV, MHV, or PEDV (26, 59). It should be noted though that for these coronaviruses, this effect has not yet been demonstrated to depend directly on the catalytic activity of PL2pro, meaning that secondary effects of the infection on cellular ubiquitination levels cannot be formally excluded as a (partial) explanation for the observed decrease. Considering the importance of ubiquitination as a regulatory mechanism in a multitude of cellular processes and the fact that for example innate immune signalling is both positively and negatively regulated via ubiquitination (for a review see (101)), it would seem counterproductive for the virus to have such a dramatic and seemingly random effect on the level of ubiquitination. However, as in many aspects relating to virus replication, timing might be of the essence in this case. One can for example imagine that target specificity is of greater importance at early time-points after infection than at later time-points, when the infection is already well underway and only limited additional time is required to reach peak viral titers. Indeed, for MHV in a time-course experiment a general effect on ubiquitination started to be observed at about 12 hours post infection (59), which is approximately when peak viral titers are reached (102). The general effect of EAV on protein ubiquitination levels was observed at 10 hours post infection (Figure IV-5D), which is only a
few hours away from the moment at which peak viral titers are reached. The effect on ubiquitination levels at earlier time points has however not yet been assessed for EAV. A possible explanation for the observed promiscuity of these viral DUBs might thus be that after a certain point in infection, they become so abundant that restrictions on target specificity diminish or no longer exist. In contrast, at earlier stages the activity of these DUBs might still be kept in check by for example protein-protein interactions, localization, and lower concentrations. Additional experimental work will have to establish whether the promiscuity of viral DUBs changes in the course of infection. If this indeed turns out to be the case, efforts can be directed at delineating what the specific early targets of these enzymes are.

A recent discovery that might have interesting implications for the target specificity of arterivirus PLP2 is the presence of a programmed ribosomal frameshift (PRF) site in the nsp2-coding region of all arteriviruses except EAV (103). In a substantial fraction of translation events, this PRF (which can be either -1 or -2) produces two truncated forms of nsp2: nsp2TF (~20%) and nsp2N (~7%). Both these proteins encompass the PLP2 domain but only nsp2TF contains a (predicted) transmembrane domain. The fact that nsp2N is possibly not anchored to membranes may have implications for its subcellular localization and probably also its substrate access. Interestingly, also nsp2TF was shown to not localize to replication and transcription complexes but to an alternative location that awaits further experimental clarification (103). The EAV nsp2-coding region does not contain a PRF site, yet in some cell types a small protein product encompassing the PLP2 domain but not the downstream transmembrane domain was observed during infection ((104) and our unpublished data). The relevance of this observation needs to be further investigated, but EAV might thus have evolved an alternative mechanism to produce a PLP2-containing cytosolic protein, as possibly achieved with the PRF in other arteriviruses. Taken together, the alternative localization and protein-protein interactions of these truncated forms of nsp2 might create possibilities for PLP2 to interact with different substrates. It would therefore be interesting to investigate the importance of this PRF event for the immune evasive activity and target specificity of PLP2.

**Identifying the targets of arterivirus PLP2 DUB activity**

Assuming that they exist, the search for specific targets of arterivirus PLP2 will likely be a daunting task and confirmation of the involvement of any target will probably require a combination of different experimental approaches. To date, arterivirus PLP2 and coronavirus PL2pro have been shown to decrease the level of ubiquitination of a number of suspected targets in ectopic expression and *in vitro* assays ([Table VII-1](#)) ([Chapter 2](#) and (25, 26, 53, 61, 105)). However, considering the promiscuous nature of these DUBs, such an effect could likely be demonstrated for virtually any ubiqui-
tinated substrate under these experimental conditions. Ideally, the search for specific substrates should therefore focus on endogenous proteins at early time-points during infection. For example, one could envision an unbiased proteomics-based approach in which the level of ubiquitination of all cellular proteins is assessed during infection. Enrichment for ubiquitinated proteins can be achieved in different ways. For example, one can perform immunoprecipitations (IPs) targeting endogenous ubiquitin with ubiquitin-specific antibodies or antibodies specific for peptides harbouring the double-glycine motif that remains after trypsin digestion of a ubiquitinated substrate (106). Alternatively, experiments can be performed in cell lines stably expressing epitope-tagged ubiquitin, which can then be used for IPs as has been described for the ubiquitin-like protein SUMO (107). Combining such approaches with stable isotope labelling with amino acids in cell culture (SILAC) (108) will allow for a comparison between the effects of wild-type and PLP2 DUB-negative viruses.

It needs to be noted though, that any differences in ubiquitination levels revealed by such a comparison cannot be directly attributed to PLP2 DUB activity, since for example the ubiquitination status of one protein can affect that of another protein downstream in a signalling cascade. Additional experimental work will therefore be needed to establish a direct interaction between PLP2 and the identified putative substrate. One approach would be to perform a co-IP, as was successfully used for the identification of TRAF3 as a target of the cellular deubiquitinase DUBA (109). However, since these interactions will likely be of a transient nature, as is often the case with protease-substrate interactions, confirming their existence in this manner might prove to be very difficult. One often-used trick to increase the duration of protease-substrate interactions is to mutate catalytic residues. However, in the context of an infection this will not be possible because of the dependence of the virus on PLP2 proteolytic activity for replicase maturation. Alternatively, this approach can be used in the context of ectopic expression, which will however increase the risk of obtaining false-positive results. Another option to potentially increase the sensitivity of co-IPs during infection is to cross-link interacting proteins with for example formaldehyde (110).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Target</th>
<th>Type of experiment</th>
<th>References</th>
</tr>
</thead>
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<tr>
<td>EAV PLP2</td>
<td>RIG-I</td>
<td>Ectopic expression</td>
<td>Chapter 2</td>
</tr>
<tr>
<td>PRRSV PLP2</td>
<td>IkBα</td>
<td>In vitro</td>
<td>(53)</td>
</tr>
<tr>
<td>MHV PL2pro</td>
<td>TBK1, IRF3</td>
<td>Ectopic expression</td>
<td>(25, 61)</td>
</tr>
<tr>
<td>PEDV PL2pro</td>
<td>RIG-I, STING</td>
<td>Ectopic expression</td>
<td>(26)</td>
</tr>
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<td>HCoV-NL63 PL2pro</td>
<td>RIG-I, TBK1, IRF3, STING</td>
<td>Ectopic expression</td>
<td>(105)</td>
</tr>
</tbody>
</table>
When a direct interaction of PLP2 with a putative substrate would be confirmed, future research should focus on elucidating the effects of its deubiquitination during infection. While to date most research concerning both arteri- and coronavirus DUBs has focused on their role in innate immune evasion, it should not be forgotten that ubiquitin plays a role in a variety of cellular processes, many of which might somehow influence viral replication and might thus be relevant targets for viral DUBs. Especially the ubiquitin-proteasome system might prove of particular importance, for example in directing the degradation of viral proteins, which could in turn be prevented by viral DUBs. An example of such a role for a viral DUB is provided by the TYMV protease, which prevents the proteasomal degradation of the viral polymerase via its deubiquitination (31).

**ASSESSING THE ROLE OF ARTERIVIRUS PLP2 DEISGYLATING ACTIVITY**

**Viral deISGylating enzymes**

Whereas eukaryotic DUBs are generally considered (with some exceptions (111, 112)) to be specific for either ubiquitin or a ubiquitin-like protein, several of their viral counterparts have been found to target both ubiquitin and ISG15. Since the latter has been shown to inhibit replication of a large variety of viruses (for a review, see (113)), it is not surprising that several viruses have evolved enzymes with deISGylating activity. Examples of viral proteases that were shown to harbour both DUB and deISGylating activity include coronavirus PL2pro (23, 27, 28, 60) and nairovirus OTU (1, 32, 33). Of these, the deISGylating activities of SARS-CoV PL2pro (114) and the OTU protease encoded by the nairovirus Crimean-Congo hemorrhagic fever virus (CCHFV) (32, 33) have been clearly demonstrated using in vitro assays with 7-amino-4-methylcoumarin (AMC)-tagged ISG15. Interestingly, these assays show that the specificity constant of SARS-CoV PL2pro for ISG15-AMC is approximately one order of magnitude larger

<table>
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<th>Enzyme</th>
<th>Substrate</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ub-AMC</td>
<td>hISG15-AMC</td>
</tr>
<tr>
<td>SARS-CoV PL2pro</td>
<td>1.31 x 10^4</td>
<td>ND</td>
</tr>
<tr>
<td>SARS-CoV PL2pro</td>
<td>1.98 x 10^4</td>
<td>6.02 x 10^5</td>
</tr>
<tr>
<td>EAV PLP2</td>
<td>1.7 x 10^4</td>
<td>ND</td>
</tr>
</tbody>
</table>

**Abbreviations:** AMC, 7-amino-4-methylcoumarin; hISG15, human interferon-stimulated gene-encoded protein of 15 kDa; ND, not determined; Ub, ubiquitin.
than that found for Ub-AMC, while the specificity constants of CCHFV OTU for both substrates lie within the same order of magnitude (Table VII-2). These results seem to suggest that the deISGylating activity of SARS-CoV PL2pro is more important than its DUB function, but some caution should be taken in the interpretation of these findings. Notably, these AMC-linked substrates differ significantly from the naturally occurring substrates of these enzymes in that they do not contain isopeptide bonds. Indeed, there is evidence to suggest that the activity of SARS-CoV PL2pro towards Lys48-linked tetra-ubiquitin chains is comparable to its activity towards ISG15-AMC (114). Another study showed that the quantitatively assessed in vitro activity of CCHFV OTU towards a Lys63-linked di-Ub is over one order of magnitude higher than its activity towards Ub-AMC (32). Additional characterization of the enzymatic activity of DUBs and deISGylating viral enzymes will likely benefit from the use of substrates that more closely resemble naturally occurring isopeptide-linked substrates, such as the fluorescence polarization-based ubiquitin(-like) substrates described in Chapter 3 (44, 115) and polyubiquitin substrates. Another important consideration is the lack of amino acid sequence conservation of ISG15 between species, which stands in stark contrast to the almost complete conservation of the ubiquitin sequence across eu-karyotes (Figure VII-4A and B). Notably, in vitro assays with the CCHFV OTU protease revealed a distinct preference for human over mouse ISG15 (116).

Is arterivirus PLP2 also a deISGylating enzyme?

Although arterivirus PLP2 has also been suggested to act both as a DUB and a deISGylating enzyme, the latter activity remains poorly characterized and conflicting data on this subject have been reported. The (putative) deISGylating activity of EAV and PRRSV PLP2 has been demonstrated using ectopic expression systems and in infected cells (1, 54). However, the possibility that the observed decrease in the level of ISGylation was the result of an indirect or nonspecific effect of the ectopic expression of PLP2 or infection cannot be formally excluded. In addition, preliminary in vitro experiments with EAV PLP2 using human ISG15-TAMRA as a substrate revealed only very limited proteolytic activity (personal communication: P. Geurink, NKI, Amsterdam). Considering the narrow host range of arteriviruses, we reasoned that the arterivirus PLP2 domains might have evolved to preferentially accommodate only the ISG15 encoded by their respective hosts. In support of this idea, a structural model of EAV PLP2 in complex with human ISG15, based on the crystal structure of EAV PLP2 in complex with ubiquitin (PDB ID: 4IUM), shows that some non-conserved residues indeed localize to the area that potentially forms the PLP2-ISG15 interface, thereby supporting the hypothesis that species-specificity matters (Figure VII-4C and D). Notably, we have been able to show in vitro complex formation between EAV PLP2 and an equine ISG15 suicide probe (see below), which at least suggests the pos-
However, according to a recent publication by Deaton et al. (2014), in vitro activity of PRRSV PLP2 towards human ISG15-AMC and, strikingly, also human and porcine proISG15 (i.e. ISG15 with a short C-terminal extension) could hardly be demonstrated, suggesting that the lack of cleavage in this particular experiment was not due to the use of non-porcine ISG15 (20). It needs to be noted though, that an approximately 14- to 20-fold lower PLP2 concentration was used for the porcine compared to the human proISG15 substrate, and a reason for
this difference was not provided. Finally, it is conceivable that the PLP2s encoded by EAV or PRRSV inherently differ from each other in their activity towards ISG15. Taken together, the postulated delSGylating activity of arterivirus PLP2 has not yet been firmly established and additional work on this subject is required.

Further assessment of the role of arterivirus PLP2 delSGylating activity should preferably start with the in vitro characterization of this activity using (isopeptide-linked) ISG15 substrates derived from the appropriate species. Once delSGylating activity would indeed have been demonstrated, efforts can be directed at establishing the role of this activity during infection. To this end, one approach would be to specifically decrease the delSGylating activity through targeted mutagenesis and assess the effect of such mutations on viral replication, similar to what was described for EAV PLP2 DUB activity in Chapter 4. Several PRRSV PLP2 mutants with decreased delSGylating activity (assessed in cell culture using monkey ISG15) have been described previously (54). However, when transferred to the full-length clone most of these mutants proved nonviable, possibly due to problems with polyprotein maturation, and the effect on DUB activity of these mutations was not further assessed. The rational design of mutations that only affect delSGylating activity would benefit from the availability of a three-dimensional structure of PLP2 in complex with ISG15. As briefly mentioned above, it is possible to produce a covalent complex of EAV PLP2 and an equine ISG15 suicide substrate in sufficient quantities to screen for crystallization and initial crystallization trials have been performed (unpublished data). Future efforts should now focus on obtaining diffraction-quality crystals that will open the way for a thorough assessment of arterivirus PLP2 delSGylating activity during infection.

CONCLUDING REMARKS

The multifunctional nature of arterivirus PLP2 is a fascinating but by no means unique feature of this protease. In fact, many +RNA virus proteases have been found to target both viral and cellular substrates (for an overview, see Table VII-3). For example, the PRRSV nsp4 protease was recently shown to be responsible for the cleavage of the innate immune signalling factor NEMO (78). Similarly, several flavivirus main proteases have been shown to cleave innate immune signalling factors such as MAVS and STING (79, 80, 87, 88). Most work on multifunctional viral proteases has been reported for the picornaviruses, whose main and accessory proteases have been shown to target among others the innate immune signalling factors MAVS, MDA5, and RIG-I (82-86). Their host substrate-directed proteolytic activity is however not limited to proteins involved in innate immunity. For example, also several host proteins involved in transcription and translation are cleaved by picornaviral proteases (117-142).
assuming that (especially in the case of the main proteases) the primary role of these viral enzymes is in replicase maturation, and that the host-directed activities were acquired at a later stage, one might wonder how the latter could have evolved without negatively affecting the activity of the protease towards its viral substrate. It seems difficult to reconcile the need for strict specificity towards (often multiple) sites within the replicase polyprotein, with the development of activity towards unrelated host proteins. A possible solution for this apparent problem is to exploit different regions of the protease to support an interaction with viral versus host substrates. In the case of arterivirus PLP2 for example, it seems that its interaction with ubiquitin relies on residues that are not involved in the interaction with the viral polyprotein (Chapter 4). Crystal structures of other multifunctional viral proteases in complex with their respective cellular substrates will be needed to answer the question whether such differential sites of interaction are a common feature. If this indeed turns out to be the case, this opens up possibilities for the separation of the activities towards their viral and host substrates, as was done for EAV PLP2.

Cleavage of MAVS by the hepatitis C virus main protease can be almost completely abrogated by a single amino acid substitution within the cleavage site (79, 80). Considering that such small changes can protect the host against virus-induced cleavage, it seems rather odd that so many (innate immune signalling) proteins remain targets of these multifunctional proteases. This implies that the selective pressure on the host to escape from these viral proteases is not strong enough to drive evolution of these cellular targets. An alternative explanation for the apparent lack of adaptation of these cellular targets is the possibility that the host actually benefits from the imposed brake on innate immune activation provided by the virus. The activation of immune signalling needs to be carefully balanced to prevent immunopathology, and both the virus and the host will likely fare better when this balance is not disturbed too much. These considerations do not readily apply to viral DUB activity because of the importance of the LRLRGG sequence for the correct functioning of the entire ubiquitin conjugation system. DUB activity is thus a viral property from which the host can hardly escape, which is likely one of the reasons why various virus groups have acquired this ability.

In conclusion, the results described in this thesis have provided novel insights in the structural features of arterivirus PLP2, in addition to confirming its putative DUB activity and establishing its role in innate immune evasion. Future research should now focus on identifying the host and/or viral protein target(s) of this intriguing protease and on a further assessment of its potential use in the design of improved arterivirus vaccines.
Table VII-3. Overview of multifunctional +RNA viral proteases.

<table>
<thead>
<tr>
<th>Order</th>
<th>Family</th>
<th>Genus</th>
<th>Species</th>
<th>Protease</th>
<th>Cellular substrate(s)</th>
<th>Cellular process</th>
<th>References</th>
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<td>Nidovirales</td>
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<td>Arterivirus</td>
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<td>NEMO</td>
<td>Innate immunity</td>
<td>(78)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>EAV, PRRSV</td>
<td>PLP2</td>
<td>Ubiquitin, ISG15</td>
<td>Innate immunity</td>
<td>Chapter 2, (1, 53, 54)</td>
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<td></td>
<td></td>
<td>LDV, SHFV</td>
<td>PLP2</td>
<td>Ubiquitin</td>
<td>Innate immunity</td>
<td>Chapter 2</td>
</tr>
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<td>Coronaviridae</td>
<td>Alphacoronavirus</td>
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<td>PL1pro</td>
<td></td>
<td>Ubiquitin</td>
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<td>(21)</td>
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<td></td>
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<td>(24, 60, 145)</td>
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<td>(25, 61)</td>
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<td>(27, 28)</td>
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<td>(30)</td>
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<td></td>
<td></td>
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<td></td>
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<td>(117-120)</td>
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<td></td>
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<td>3Cpro</td>
<td></td>
<td>NEMO/IKKγ</td>
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<td>(81)</td>
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<td></td>
<td></td>
<td>FMDV</td>
<td>3Cpro</td>
<td></td>
<td>Histone H3</td>
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<td>(146, 147)</td>
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<td>3Cpro</td>
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<td>RIG-I</td>
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<td>(83)</td>
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<td>EVB, RV</td>
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<td>Dystrophin, KB</td>
<td>Cytoskeletal integrity</td>
<td>(148, 149)</td>
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<td>MDAS, MAVS</td>
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<td>TBP</td>
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<td>EV B/C, RV</td>
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<td>elf4G, PABP</td>
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<td>IκBα</td>
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<td>Cstf-64</td>
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Table VII-3. Overview of multifunctional +RNA viral proteases. (continued)

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<th>Species</th>
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<td>RIG-I</td>
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<td>Vesivirus</td>
<td>FCV</td>
<td>3CL&lt;sup&gt;pro&lt;/sup&gt;</td>
<td>PABP</td>
<td>Translation (160)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Flavivirus</td>
<td>DENV</td>
<td>NS2B3pro</td>
<td>STING</td>
<td>Innate immunity (87, 88)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hepacivirus</td>
<td>HCV</td>
<td>NS3/4A</td>
<td>TRIF</td>
<td>Innate Immunity (89, 90)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>HCV, GBV-B</td>
<td>NS3/4A</td>
<td>MAVS</td>
<td>Innate Immunity (79, 80, 161, 162)</td>
</tr>
</tbody>
</table>

1 Classification based on ICTV Master Species List 2012 version 2.
2 DENV, dengue virus; EAV, equine arteritis virus; EMCV, encephalomyocarditis virus; EVA/B/C, enterovirus A/B/C; FCV, feline calicivirus; FMDV, foot-and-mouth disease virus; GBV-B, GB virus B; HAV, hepatitis A virus; HCV, hepatitis C virus; HPeV-1, human parechovirus 1; LDV, lactate dehydrogenase-elevating virus; MERS-CoV, Middle East respiratory syndrome coronavirus; MHV, murine hepatitis virus; PEDV, porcine epidemic diarrhea virus; PRRSV, porcine reproductive and respiratory syndrome virus; RV, rhinovirus; SARS-CoV, severe acute respiratory syndrome coronavirus; SHFV, simian hemorrhagic fever virus; TGEV, transmissible gastroenteritis virus; TYMV, turnip yellow mosaic virus.
3 2A<sup>pro</sup>, 2A protease; 3C<sup>pro</sup>, 3C protease; 3CL<sup>pro</sup>, 3C-like protease; L<sup>pro</sup>, leader protease; NS2B3pro, nonstructural protein 2B-3 protease; NS3/4A, nonstructural protein 3 and 4A; nsp4, nonstructural protein 4; PLP2, papain-like protease 2; PL1/2pro, papain-like protease 1 or 2; PRO, protease.
4 CREB, cAMP response element-binding protein; CstF-64, cleavage stimulation factor 64; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; eIF4A/4G/5B, eukaryotic initiation factor 4A/4G/5B; G3BP-1, GTPase activating protein (SH3 domain) binding protein 1; IκBα, inhibitor of nuclear factor kappa B alpha; IKKγ, inhibitor of nuclear factor kappa B kinase gamma; IRF7, interferon regulatory factor 7; ISG15, interferon-stimulated gene-encoded protein of 15 kDa; K8, cytokeratin 8; La, La autoantigen; MAP-4, microtubule-associated protein 4; MAVS, mitochondrial antiviral signaling; MDAS, melanoma differentiation-associated protein 5; NEMO, nuclear factor kappa B essential modulator; Nup62/98/153, nucleoporin 62/98/153 kDa; Oct-1, octamer-binding transcription factor 1; PABP, poly(A) binding protein; PTB, polypyrimidine tract-binding protein; RIG-I, retinoic-acid inducible gene-encoded protein I; SS-B, Sjögren Syndrome antigen B; STING, stimulator of interferon genes; TBP, TATA-binding protein; TRIF, TIR-domain-containing adaptor protein inducing interferon beta.
5 Hypothesized or experimentally confirmed.
REFERENCES


