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Summary

ARTERIVIRUS PLP2
An OTU deubiquitinase that counteracts innate immunity

Despite their limited size, viruses can have a massive impact on their hosts on both the individual and population level. This becomes even more fascinating when taking into consideration the fact that viruses are in essence lifeless entities, only capable of replicating within a permissive host. A proper understanding of the viral "life cycle" can therefore only be obtained when, in addition to the replication mechanism of the virus itself, also the virus-host interactions are elucidated.

In the case of mammalian positive-stranded (+) RNA viruses, proteases can fulfil an important role in the interplay between the virus and its host. This group of viruses often produces (part of) their proteins in the form of a polyprotein, that is subsequently cleaved into functional subunits by viral proteases residing within these polyproteins. In addition to playing this vital role in polyprotein maturation, these proteases can exert other functions by also cleaving host factors. The work described in this thesis demonstrates how an arterivirus protease (PLP2) interacts with the cellular ubiquitin system and thereby inhibits activation of the innate immune response.

Formally, the arterivirus family currently encompasses four members, including the equine arteritis virus (EAV) and the porcine reproductive and respiratory syndrome virus (PRRSV). Especially the latter poses a great burden on swine-farming industries worldwide, through the loss of piglets and reduced fertility of sows. Vaccines are available for both these viruses, although their efficacy is suboptimal. This is thought to be caused in part by the capability of these viruses to counteract the innate immune response, which in turn prevents full-blown activation of the adaptive immune response.

The replication of arteriviruses commences with the production of two partly overlapping polyproteins, which are the precursors of the viral nonstructural proteins (nsps). These polyproteins are co- and posttranslationally cleaved into their respective functional subunits by three to five internal proteases. Together, the nsps form the viral replication- and transcription complex, which is responsible for the replication of the viral genome and the transcription of subgenomic messenger RNAs. The proteolytic cleavage of the viral polyproteins is a highly regulated process and mutations of the proteases or cleavage sites involved are generally not well tolerated. One of the proteases that are responsible for the maturation of the arterivirus replicase polyproteins is papain-like protease 2 (PLP2), which is located in nsp2 and performs the cleavage between nsp2 and nsp3.

The innate immune response constitutes the first line of defence against invading pathogens and relies on the recognition of pathogen-associated molecular patterns by specific pattern-recognition receptors. Activation of the innate immune response ultimately leads to the transcription of genes encoding type I interferons and other pro-inflammatory cytokines, which together are responsible for the induction of an antiviral state in both infected and neighbouring cells and the stimulation of the adaptive immune response.

The activation of the innate immune response is strictly regulated, among others via the process of ubiquitination, i.e. the conjugation of the small ubiquitin protein to specific target proteins. This conjugation can lead to the proteasomal degradation of the ubiquitinated substrate, but can also support certain protein-protein interactions. Ubiquitination is a posttranslational modification that is particularly suitable for the regulation of signal transduction routes since it is readily reversible through the action of deubiquitinating enzymes (DUBs).

Comparative sequence analyses performed by Makarova *et al.* (2000) have shown that arterivirus PLP2 resembles proteases belonging to the OTU family of DUBs. Later, Frias-Staheli *et al.* (2007) have demonstrated that ectopic expression of EAV or PRRSV PLP2 in cell culture leads to a decrease of the levels of ubiquitinated proteins. Considering the importance of ubiquitination for the activation of innate immunity, it seemed likely that the putative DUB activity of arterivirus PLP2 plays a role in counteracting this response. However, the essential role of PLP2 in the maturation of the viral polyproteins precluded its straightforward deletion or inactivation, and thus made it difficult to provide proof for this hypothesis.

For this reason, our first aim was to confirm the DUB activity of arterivirus PLP2 and to further characterize its putative role in the evasion of the innate immune response. In **Chapter 2**, we have firmly established the DUB activity of EAV PLP2 using *in vitro* cleavage assays and, using transfection experiments, we have shown that this activity is likely conserved across the arterivirus family. Furthermore, we have demonstrated that the ectopic expression of arterivirus PLP2 leads to a decreased expression of a luciferase-reporter gene under control of the interferon beta promoter. The pattern-recognition receptor RIG-I is an important, ubiquitin-regulated factor in the innate immune response. By means of a transfection experiment we were able to show that arterivirus PLP2 decreases the ubiquitination of RIG-I in cell culture. These data supported the hypothesis that the DUB activity of arterivirus PLP2 is important for the inhibition of the innate immune response.

Due to their essential role in the replication of mammalian +RNA viruses, proteases have proven to be a good target for the development of antiviral compounds. Since in addition to arteriviruses, several other viruses encode proteases with DUB activity, we investigated whether this activity could be exploited to identify potential antiviral compounds. **Chapter 3** includes the result of a fluorescence polarization-based *in vitro* screen, by which from a library of 335 compounds, five were found to inhibit the DUB activity of EAV PLP2. Of these, two turned out to also inhibit replication of a GFP-encoding EAV reporter virus in cell culture to a considerable extent. It thus seems that it is indeed possible to identify antiviral compounds using a DUB-based screen, although additional work is required.

To establish the role of PLP2 DUB activity during infection, it was important to separate this activity from its essential function in the maturation of the viral replicase polyproteins. In collaboration with Canadian structural biologists, we therefore solved the crystal structure of EAV PLP2 in complex with ubiquitin (**Chapter 4**). From the three-dimensional structure it was evident that PLP2 indeed belongs to the OTU family and that the interaction with ubiquitin partly relies on a surface of PLP2 that is distant from the active site. This enabled us to design mutations that specifically interfered with the interaction with ubiquitin, without disrupting the activity of PLP2 towards the viral polyproteins. Using these mutations, we were able to demonstrate that the DUB activity of arterivirus PLP2 is indeed important for the inhibition of innate immunity during infection.

Chapter 5 encompasses a small-scale vaccination experiment in horses in which we tested whether an EAV vaccine that lacks PLP2 DUB activity (DUB-) provides better protection against challenge with a heterologous EAV isolate than a vaccine virus in which PLP2 DUB activity is still intact (DUB+). This experiment showed that both vaccine viruses are replication competent. However, we did not observe a clear difference in the level of protection between the two vaccine viruses. The fact that the DUB+ vaccine virus already provided a very high level of protection against a challenge infection in this experiment, may have been the reason why it was difficult to detect any further improvement. Additional experiments are needed to establish whether the inactivation of PLP2 DUB activity yields better results in a different experimental setting.

In addition to arteriviruses, both nairoviruses (-RNA) and tymoviruses (+RNA) encode OTU DUBs. **Chapter 6** presents a concise structural and functional comparison between these viral enzymes. While both arteri- and nairovirus DUBs seem to function in evading the innate immune response, tymovirus DUBs appear to be responsible for

preventing the proteolytic degradation of the viral RNA polymerase. The most striking structural difference between these enzymes is the fact that the catalytic core of the tymovirus DUB seems to be incomplete in comparison with that of arteri- and nairoviruses.

The work described in this thesis provides novel insights into the structural and (multi) functional characteristics of arterivirus PLP2. We have demonstrated for the first time the importance of a viral DUB in the evasion of innate immunity in the context of an infection. The acquired knowledge can now be applied to the design of improved arterivirus vaccines and studies of other viral DUBs, including those encoded by the zoonotic coronaviruses that cause severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS).