Chapter 7

General discussion

THE NEED FOR ANTIVIRAL THERAPIES AGAINST PATHOGENIC CORONAVIRUS INFECTIONS

The 2003 SARS-CoV outbreak, which had an immense global impact and almost led to a pandemic, and the ongoing MERS-CoV outbreak illustrate it is important to be prepared for emerging pathogenic coronaviruses. The re-emergence of SARS-CoV or the zoonotic transfer of other animal coronaviruses like MERS-CoV to humans remains a serious public health concern. The preparedness for these outbreaks should obviously include the availability of antiviral intervention strategies. The number of confirmed MERS cases that have been reported is relatively low (94 cases and 46 deaths till August 2013), but the mortality rate in this group of patients is alarmingly high (~50%). Moreover, in view of the distribution of these cases in time and over distant geographical locations, it is now widely assumed that the actual number of human infections could be much higher. A major factor that may contribute to an underestimation of that number obviously is the possibility of mild or asymptomatic infections, but also inadequate surveillance, lack of proper diagnostic procedures and/or the political and social climate in the Middle East may play a role. Therefore, the true number of cases and the risk of the virus spreading to other regions might be underestimated. Nevertheless, in-depth analysis of the small MERS clusters described so far suggests that the currently circulating MERS-CoV strains do not have pandemic potential [398].

The SARS outbreak triggered the rapid development of vaccine candidates (reviewed in [399]) and one of these even entered a phase I clinical trial [400] within a relatively short period of time, but still 1.5 years after the start of the outbreak. Since the 2002/2003 global SARS outbreak was controlled within about 4 months and only a few additional cases have been reported since then, no further clinical trials were conducted. Considering the time required to test and register a vaccine, it is important to develop alternative intervention strategies (e.g. pan-coronavirus inhibitors) as a first line of defence, to combat any new pathogenic coronavirus that might emerge in the future.

A better understanding of coronavirus replication and the complex interplay between the abovementioned viruses and their host should provide starting points for the development of such antiviral strategies. They may also offer possibilities to limit or circumvent the problem of antiviral drug resistance, a common problem when using compounds that directly target RNA viruses, due to their high mutation rate and rapid adaptation. Therefore, there is a growing interest in druggable host targets to block virus replication [401], as drug resistance is less likely to develop when cellular rather than viral functions are targeted (reviewed in [130, 131]). **Chapters 2, 3 and 5** of this thesis shed some light on the interplay between nidoviruses and the host cell. The different approaches used in these studies, each with their own benefits and limitations, identified a variety of host factors that can influence nidovirus replication. Studies using isolated,

functional replication and transcription complexes (RTCs) from arterivirus-infected cells contributed to the identification of antiviral compounds and proviral host factors. An siRNA screen identified kinases and various other host factors that affect SARS-CoV replication. Finally, the cyclophilins (Cyps), a promising class of host targets for antiviral therapy, were shown to play a role in the replication of both arteri- and coronaviruses (**Chapters 3, 4 and 6**). Below, a selection of these host factors, their functions in the host cell and their role in nidovirus replication will be discussed in more detail.

Characterisation of replication complexes isolated from infected cells

The isolation of viral replication complexes (RCs) from infected cells and the development of *in vitro* assays (IVRAs) to analyse their RNA synthesising activity have provided mechanistic insights into the replication of several +RNA viruses, including human pathogens like poliovirus [259, 264, 268, 402], West Nile virus [227, 244], Japanese encephalitis virus [403], and HCV [254, 404]. The development of such assays for model viruses like Sindbis virus [228], brome mosaic virus [261] and Flock house virus [405] has contributed to our understanding of +RNA virus replication in general. Besides providing information on viral RNA synthesis mechanisms and the role of membrane structures in replication, also the involvement of host proteins in RNA synthesis [264, 406] was established and the mode of action of certain inhibitors was resolved, for example in the case of Gliotoxin, which was shown to be an inhibitor of the poliovirus polymerase $3D^{pol}$ [407].

For nidoviruses, several previous attempts to study RNA synthesis using RTCs isolated form infected cells [236, 239, 240] resulted in barely detectable activities *in vitro*. **Chapter 2** describes a robust assay to study nidovirus RNA synthesis *in vitro*. A crude cytoplasmic fraction from cells infected with the prototype arterivirus EAV, contained RTCs that were able to synthesise EAV genomic RNA and all sg mRNAs *in vitro*. Further subcellular fractionation yielded a membrane fraction that contained these functional RTCs. Strikingly, their RNA synthesising activity was strongly dependent on a cytosolic host factor that was not physically associated with the RTCs after their isolation. In parallel to this study of the arterivirus RTCs, the same phenomenon was observed for RTCs isolated from SARS-CoV-infected cells [53]. This host factor could be partially purified from the cytosol of (uninfected) HeLa cells by size-exclusion chromatography and was found to have a native mass of 59-70 kDa. Several other chromatographic methods were applied in attempts to further purify and identify the host factor necessary for EAV RNA synthesis, and it still remains to be seen whether it is the same factor that is crucial for SARS-CoV replication. Its identification was hampered by the fact that all protein fractions purified need to be assayed for their ability to reconstitute the activity of pelleted (inactive) EAV RTCs, and that the preparation of these complexes is laborious and complicated, in particular since they lose their RNA-synthesising activity relatively quickly. Comparative quantitative mass spectrometry (in collaboration with the LUMC

Biomolecular Mass Spectrometry Facility) has now narrowed down the list of candidates to six proteins (de Wilde *et al*., unpublished data), but unfortunately the exact identity of this host protein remains to be established. Only a small amount of this factor is needed for EAV RTC activity, since a 50-fold diluted HeLa cell lysate or chromatography fractions containing almost undetectable amounts of protein can still reconstitute viral RNA synthesis (de Wilde *et al*., unpublished data). The need for a host factor that apparently has no permanent physical interaction with the viral RTC was also observed for poliovirus RNA synthesis in a study by Barton *et al*. [264], and the identity of this factor is currently still unknown. I hypothesise that this factor modifies (by e.g. phosphorylation) and activates a host or viral protein within the RTC that is required for efficient RNA synthesis. Its unequivocal identification and functional characterisation, using siRNAs, chemical inhibitors and heterologous expression and purification is currently ongoing.

Viral RNA synthesis is a logical target for developing antiviral strategies and inhibitors of this step of the replicative cycle have been successfully developed for several +RNA viruses (reviewed in [318, 408, 409]). *In vitro* assays have been used to analyse nidovirus RNA synthesis but also for screening for compounds that inhibit RNA synthesis, as exemplified by the identification of CsA (**Chapter 3**) and Zn^{2+} [159]. An advantage of screening for inhibitors in an *in vitro* system is that cellular uptake does not pose any limitations, which allows the identification of (cell-impermeable) lead compounds that would have been missed in cell-based screens. A disadvantage of this approach is that it would probably not identify inhibitors that need to be metabolised first, like Ribavirin. Another drawback is that potential cytotoxic effects caused by the compound are not detected at an early stage. It obviously remains important to test inhibitors that are identified *in vitro*, in cell-based assays to evaluate their toxicity and efficacy (see also **Chapter 3**).

In conclusion, the biochemical studies and *in vitro* activity assays provided more insight into the composition and characteristics of the nidovirus RTC and form a good basis for more in-depth studies, for example on their composition and the function and origin of the associated membranes. This would require more sophisticated – thus far unsuccessful – RTC purification strategies in combination with for example mass-spectrometry.

SYSTEMS VIROLOGY AND ITS POTENTIAL TO IDENTIFY HOST FACTORS INVOLVED IN CORONAVIRUS REPLICATION

The application of systems biology approaches in virology (systems virology) has provided a wealth of information on the role of individual proteins and cellular pathways in the replication of RNA viruses (for a recent review, see [410]). This relatively new, interdisciplinary field focuses on the complex virus-host interactions that occur within

the cell or even in the whole organism and aims to provide an unbiased perspective. The applied techniques include transcriptomics (the analysis of gene expression profiles in infected cells), metabolomics (metabolic changes induced during infection), proteomics (to analyse changes in the cellular proteome caused by infection) and phenomics, which includes RNA interference (RNAi) screening. RNAi is a method that enables the specific degradation of cellular or viral mRNAs by transfecting small interfering RNA (siRNA) molecules that are complementary to the target RNA. This method is widely used to study virus-host interactions and it has identified numerous host genes involved in the replication of important human pathogens like WNV [334], DENV [335], HIV-1 [336], HCV [337-342] and influenza virus [338, 343, 344].

For coronaviruses a number of host proteins that affect the replicative cycle have been described previously ([321] and reviewed in [65, 320]), but large(r)-scale siRNA screens to systematically identify such factors have not been reported thus far. **Chapter 5** describes an siRNA screen to identify host cell kinases that influence SARS-CoV replication. Protein kinases are key regulators in signal transduction and control a wide variety of cellular processes [411]. Consequently they have been the subject of many studies aiming to develop therapeutic strategies for the treatment of a variety of diseases, and indeed many registered drugs have cellular kinases as their target [412, 413]. In addition, kinases have been shown to play important roles in the replicative cycle of many +RNA viruses.

The siRNA screen that is described in **Chapter 5** yielded a relatively high proportion of antiviral hits (90 of 778 factors; ~12% of all factors) for SARS-CoV, compared to human kinome-directed screens that have been performed with other viruses [341, 414, 415]. This might indicate that compared to other viruses, SARS-CoV replication is more restricted by cellular factors. However, differences in experimental set-up exist and should also be taken into account.

Pathway analysis mapped multiple hits to cellular immune responses, like interleukin (IL) signalling. Within the IL signalling pathways, IL-6 and IL-8 signalling have previously been implicated in controlling coronavirus infection and coronavirus-induced inflammation (reviewed in [65]), and the results described in **Chapter 5** (once again) emphasise their importance in SARS-CoV infection.

Several hits of our siRNA screen are part of the p38 MAPK pathway, which has also been implicated in nidovirus replication previously, as it regulates IL-6-, IL-8- and IL-10-mediated pro-inflammatory cytokine signalling [360, 361, 416]. This, for example, explains why MEK3, a key player in the p38 MAPK signalling pathway, was identified as antiviral hit for SARS-CoV. These results imply that activation of the p38 MAPK pathway limits SARS-CoV replication in cell culture and *in vivo*. Liao *et al*. showed that the avian coronavirus IBV induces IL-6 and IL-8 expression. Meanwhile, the same study showed that IBV has evolved a strategy to counteract IL-6 and IL-8 expression by inducing the

expression of dual-specificity phosphatase 1 (DUSP1), a negative regulator of p38 MAPK [362]. Although viral protein expression seemed not directly affected by IL-6 and IL-8, IBV apparently interferes with p38 MAPK signalling to limit expression of these pro-inflammatory cytokines. In contrast to IBV, MHV infection activates p38 MAPK signalling and the increased IL-6 production actually promotes MHV-specific protein synthesis and virus production [363]. Follow-up studies on the role of MAPKs in SARS-CoV infection and their link with the immune response and other pathways should provide more insight into the exact role of these pathways in coronavirus replication and pathogenesis, and in the apparently virus-specific (opposite) effects observed among coronaviruses.

The IL-17 signalling pathway that was identified as antiviral in the SARS-CoV siRNA screen, was recently found to be activated in epithelial cells during SARS-CoV and MERS-CoV infection [417]. Since this pathway plays a pivotal role in host defence responses against microbial invaders, the authors suggested that this pathway might be an interesting therapeutic target to limit coronavirus-induced cytopathicity and lung injury.

In parallel to the SARS-CoV siRNA screen described in **Chapter 5**, a similar screen using the same cell line was performed to identify cellular kinases that affect EAV replication [418]. For EAV, 38 proviral (40 for SARS-CoV) and 116 antiviral hits (90 for SARS-CoV) were identified. There was a remarkably small overlap between the hits identified for SARS-CoV and EAV, as only 5 common proviral (~12% of all hits) and 18 common antiviral (20% of all hits) hits were found. Apparently, these distantly related nidoviruses differ significantly in their interactions with the host. Nonetheless, the few common factors that have been identified (diacylglycerol kinase epsilon, suppressor of cytokine signalling 5, protein serine kinase H1, AarF domain containing kinase 4, and coatomer protein complex subunit beta-2) should be further evaluated as they could represent interesting targets for the development of broad-spectrum antiviral strategies against nidoviruses.

The application of RNAi screens was considered a revolution in genetics [419]. However, one should keep in mind that it also has drawbacks and that data must be interpreted with caution. This is illustrated, for example, by the fact that only three common hits were identified when data sets of three independent large-scale RNAi screens for host factors involved in HIV-1 replication were compared (reviewed in [420]). One of the major drawbacks of RNAi screening is that this method is relatively error prone and major concerns include the identification of false positive hits due to off-target effects, e.g. siRNAs might down-regulate multiple transcripts, siRNAs might stimulate the immune response, or exogenous siRNAs could saturate the RNAi machinery to block processing of essential cellular (mi)RNAs (reviewed in [421]). False negative hits could be observed due to insufficient knockdown of the factor that is being analysed [422]. Bearing this in mind, one should consider hits from siRNA screens only as a starting point for further analysis rather than a definitive list of host factors involved in virus replication. It also demonstrates the importance of validation experiments after the primary screen,

for example using a deconvoluted set of siRNAs targeting the same gene, lentivirus-expressed shRNAs and/or chemical inhibitors, if available for the target. A drawback of the use of chemical inhibitors is their potential lack of specificity. Overexpression studies of the identified proteins are generally expected to show an effect opposite of that found in the siRNA screen, although - depending on the endogenous level of the host factor - these effects might be limited or absent. However, the combined results of such follow-up/validation studies should increase the reliability of hit identification.

The sets of pro- and antiviral host factors that were identified for SARS-CoV provides a good starting point for in-depth analysis of nidovirus-host interactions and the cellular pathways that influence replication, which might ultimately lead to the development of host factor-directed antiviral strategies.

COP-I COATED VESICLES AND MEMBRANES IN CORONAVIRUS REPLICATION

COPB2 (or β'-COP), a subunit of the coatomer protein complex, was identified in our siRNA screen as a proviral factor for SARS-CoV, as its depletion strongly inhibited SARS-CoV protein expression and progeny titres (**Chapter 5**). Other proteins involved in the formation of COP-I coated vesicles also play a role, as depletion of COPB1 and GBF1 severely affected SARS-CoV replication. A schematic overview of (factors involved in) the formation of COP-I coated vesicles is shown in Fig. 1.

There is some controversy on the role of COP-I coated vesicles and other membrane structures in coronavirus replication. Using advanced EM analyses, our group has shown that the SARS-CoV-induced reticulovesicular network (RVN) is derived from and continuous with the ER [30, 208, 295]. In contrast, other studies implicated either the autophagic pathway [32, 54] or EDEMosomes [55] as the primary membrane source. Earlier work by our laboratory [295] and others [364-366] already suggested that (the integrity of) the early secretory pathway is important for efficient SARS-CoV replication, but its exact role remained unclear since conflicting results have been reported. Knoops *et al*. have previously shown that Brefeldin A (BFA) treatment inhibits SARS-CoV replication, but does not completely block viral RNA synthesis, as ~20% of the activity remains in BFA-treated cells, compared to untreated infected cells. Furthermore, the lack of colocalisation of SARS-CoV replicase subunits with COP-I vesicles argues against a direct association of coronavirus RTCs with these COP-I-coated vesicles [295]. Using MHV-infected cells, Verheije *et al*. showed that depletion of Arf1 and GBF1, thus blocking COP-I coated vesicle formation, and BFA treatment almost completely abolishes replication of this distantly related coronavirus. In line with the work of Knoops *et al*., co-localisation of MHV RTCs with COP-I vesicles was not observed [365]. Our data from the siRNA screen further corroborate the importance of COP-I coated vesicles and the secretory pathway

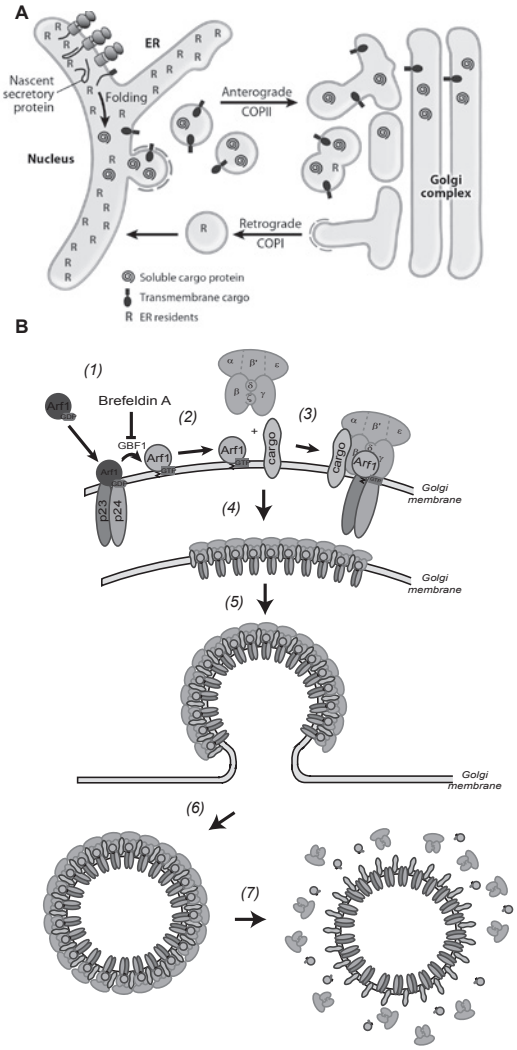

Fig. 1. (A) Model depicting bidirectional transport between the ER and Golgi compartments. After translation and folding of nascent secretory proteins, membrane cargo and soluble secretory cargo are exported from the ER in COPII-formed transport vesicles. ER-derived vesicles traffic in an anterograde direction to fuse with or to form pre-Golgi compartments. COP-I coats bud retrograde-directed vesicles from pre-Golgi and Golgi compartments to recycle vesicle components and retrieve resident proteins (R) that have escaped the ER. This rapid cycling process allows anterograde transport of secretory cargo, whereas resident proteins remain dynamically localised to early secretory compartments. Reprinted with permission from [423]. (B) Schematic overview of COP-I coated vesicle formation. Arf1-GDP binds to the cytoplasmic tails of p23/p24 hetero-oligomers (1), and is subsequently activated by the GDP/GTP exchange that is mediated by GBF1. This activation step can be blocked by BFA (2). Coatomer subsequently binds both the p24 oligomer and Arf-GTP. Cargo can be captured by the α/β′-COP subunits (3) and after coat polymerisation (4), a COP-I vesicle can pinch off the donor membrane (5,6). These vesicles uncoat due to hydrolysis of Arf-GTP to Arf-GDP (7). Based on models described in [357, 424].

for SARS-CoV replication, since we observed an almost complete inhibition of SARS-CoV replication upon depletion of COP-I coatomer components (**Chapter 5**).

COP-I-coated vesicles have also been implicated in the replication of many other RNA viruses, such as poliovirus [367, 368], other enteroviruses [353, 369-371], VSV [372], Drosophila C virus [373], and influenza A virus [344, 374] and components of the early secretory pathway have also been identified in siRNA screens for many RNA viruses [338, 344, 373, 374, 425]. These data suggest that the secretory pathway or its components are commonly used by RNA viruses. A variety of mechanisms regarding the role of the early secretory pathway have been suggested. For example, replication of the enterovirus EV11 appeared to depend on the association of COP-I with membranes [369]. On the contrary, Belov *et al*. showed that GBF1, BIG1/2 and Arf proteins, but not COP-I coatomer components, are recruited to Golgi membranes to enable RC formation [367, 368]. Hsu *et al*. reported similar observations since Arf1 and GBF1 recruit PI4KB to membranes on which RCs associate to cause a change in lipid composition. This change is favourable for the binding of other components of the RC complex [353].

To date, there is no data that suggests a direct link between COP-I vesicles and coronavirus RNA synthesis, which supports the idea that these vesicles do not contain the viral RTC but have an indirect – still to be determined – effect on coronavirus replication. COP-I vesicles might transport cargo needed for the formation or activity of viral RTCs, or the observed effects might merely be due to a general disruption of cellular endocytic transport or cellular homeostasis.

TARGETING CYCLOPHILINS TO BLOCK +RNA VIRUS REPLICATION

Cyclosporin A is a well-known immunosuppressive drug that binds to cellular cyclophilins (Cyps), yielding a Cyp-CsA complex that can inhibit calcineurin. Inhibition of calcineurin prevents the dephosphorylation and translocation of nuclear factor of activated T cells (NF-AT) from the cytosol into the nucleus (Fig. 2), which prevents the transcription of immune genes, such as IL-2 (reviewed in [279, 280, 426]). A total of 17 Cyps have been identified thus far, of which nine are targeted by CsA. Cyps are also known as peptidyl–prolyl isomerases (PPIases) and many of them have chaperone and foldase activities [279, 427] that facilitate protein folding. Cyps are involved in various signalling pathways (reviewed in [280]), and processes such as apoptosis [428] and RNA splicing [429, 430].

CsA has been reported to block the replication of a variety of RNA viruses, like hepatitis C virus [274, 299], HIV-1 [272, 432] and several others [271, 273, 275, 433]. In line with the inhibitory effect of CsA treatment, Cyps and in particular the cytosolic CypA and the ER-associated CypB have been concluded to be essential host components in the replicative cycle of many RNA viruses [271, 273, 303, 306, 307, 309, 311, 434, 435].

The work presented in this thesis shows that CsA also is a potent inhibitor of arterivirus (**Chapter 3**) and coronavirus replication (**Chapters 4 and 6**). MERS-CoV-induced cytopathology was severely reduced upon treatment with 9 μM of CsA (**Chapter 6** and unpublished observations), and SARS-CoV replication was completely blocked upon treatment with 16 μM of CsA (**Chapter 4**). Similar results were obtained for the arterivirus PPRSV (IC_{50} of 5.5 μM; complete block at 16 μM), and EAV replication appeared even five-fold more sensitive to CsA treatment (IC_{50} of 0.95 μM). Similar results were reported for feline coronavirus (FCoV)-induced cytopathology, which was blocked at 25 μM CsA. Pfefferle *et al*. have demonstrated that low micromolar concentrations of CsA block the replication of multiple coronaviruses, including SARS-CoV and HCoV-229E [277]. Noteworthy, Cyp levels may differ between test systems and therefore it is difficult to compare the sensitivities of different nidoviruses to CsA. Despite the apparently nidovirus-wide inhibitory effect of CsA, the exact mechanism by which the drug blocks nidovirus replication remains unclear. For SARS-CoV, we could not identify which specific (if any) Cyp was involved in CsA-mediated inhibition of virus replication (**Chapter 4**). This might be due to the fact that Cyp expression could not be sufficiently depleted with specific siRNAs (~25% remaining expression). The replication of SARS-CoV was not blocked by a non-immunosuppressive CsA analogue (De Wilde *et al*., unpublished data) suggesting that PPIase activity and/or direct interaction with replicase subunits might not be involved in the proviral effect of Cyps. Pfefferle *et al*. suggested that SARS-CoV nsp1 is an activator of the NF-AT signalling pathway that induces a broad and systemic dysregulation of cytokine expression [277]. Combined with our data, this would argue against a direct role for Cyps in SARS-CoV replication, but suggests that the NF-AT pathway is somehow (indirectly) involved. This model is not supported by the data obtained for FCoV replication in the feline cell line fcwf-4. The authors suggested that FCoV does not depend on a functional NF-AT signalling pathway since FCoV replication was blocked at CsA or FK506 concentrations that did not affect signalling in these cells [278]. However, these results should be interpreted with caution. At first, concentrations that were shown to block FCoV-induced cytopathicity were not tested, and secondly, the authors do not show whether or not FCoV infection activates NF-AT signalling. Therefore, it would be interesting to investigate whether FCoV induces NF-AT nuclear translocation and whether FCoV replication is sensitive to treatment with non-immunosuppressive CsA analogues. More importantly, the role of the NF-AT pathway in SARS-CoV replication should be further investigated.

In contrast to coronaviruses, replication of the arteriviruses EAV and PRRSV is inhibited by the non-immunosuppressive CsA analogue Debio-064 (**Chapter 3**). Like Debio-025, NIM811, and SCY635, this compound has a higher affinity for Cyps than CsA and lacks its undesired immunosuppressive side-effect, since NF-AT signalling is not affected by this drug. For the treatment of HCV infection, non-immunosuppressive Cyp inhibitors are

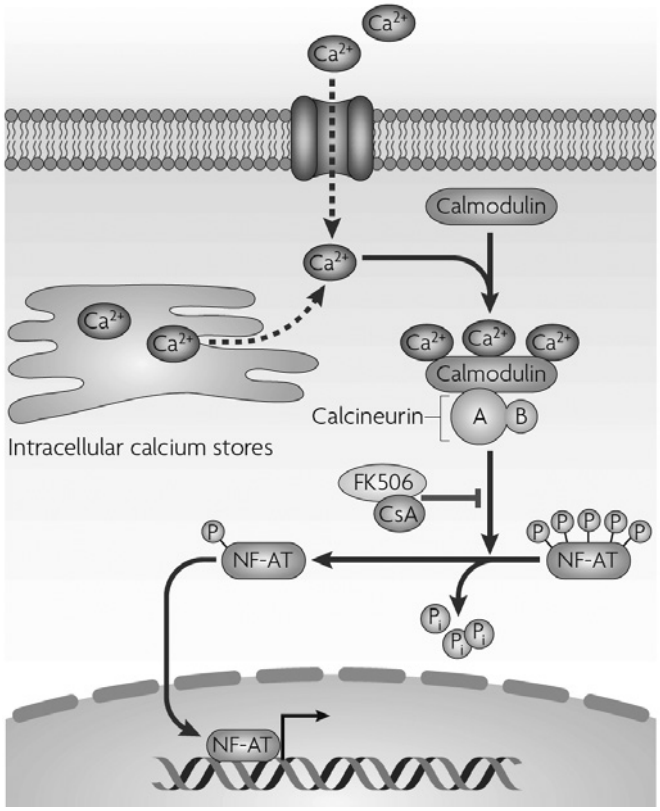

Fig. 2. The NF-AT signalling pathway. The calcineurin catalytic subunit (A), when bound to the regulatory subunit (B) and calmodulin–Ca^{2+} complex, dephosphorylates NF-AT in the cytoplasm, leading to nuclear translocation of this transcription factor and the subsequent activation of gene transcription. CsA binds to Cyps, and FK506 to FK506-binding proteins (FKBPs) and these Cyp-CsA or FK506-FKBP complexes inhibits Calcineurin activity, to reduce NF-AT dephosphorylation. Reprinted with permission from [431].

considered a promising class of antiviral compounds, and their potency was explored in phase III clinical trials, in combination with pegylated IFN and Ribavirin [436]. The non-immunosuppressive CsA analogues are not only interesting because of their therapeutic potential, they are also valuable research tools for studying the proviral mode of action of Cyps. These compounds allow us to discriminate between the direct involvement of Cyps in viral replication and indirect effects involving e.g. (dysregulation) of the NF-AT signalling pathway.

Mainly based on data obtained for HCV, different mechanisms have been proposed to explain the role(s) of Cyps in the viral replicative cycle [437]. First of all, Cyps are components of the HCV replication complex and are important for the proper folding of the HCV polymerase (NS5B) [308]. Kaul *et al*. have shown that the development of resistance against the Cyp inhibitor Debio-025 involves mutations in HCV NS5B that are close to the NS5A/NS5B cleavage site. These are thought to delay processing of the NS5A/NS5B junc-

tion, thus extending the time during which the CypA binding site in NS5B is accessible [308]. As a result, lower amounts of CypA would suffice to mediate the proper folding of NS5B and its incorporation into replication complexes. Others have postulated that the CsA treatment of infected cells leads to the depletion of CypA from the RC and that this interferes with the formation of these complexes [307, 332]. Finally, it was hypothesised that binding of the viral polymerase to the enzymatic pocket of CypA is essential for RNA synthesis [306]. Although the exact mechanism remains to be elucidated, Chatterji *et al*. suggested that the isomerase activity of CypA might be essential for NS5B function and thus for HCV replication [306], since CypA mutants that lack the PPIase activity showed a reduced binding to NS5B and PPIase-defective CypA reduced HCV replication. This hypothesis is supported by recent studies demonstrating that CsA resistance is linked to mutations in NS5B that increase its affinity for viral RNA, thus rendering the enzyme less dependent on CypA [438].

Chapter 4 describes that depletion of CypA affects EAV replication and that CypA cosediments with the membrane-associated viral RTCs. Furthermore, CsA treatment was able to prevent the cosedimentation of CypA and EAV RTCs. Although we were unable to show a direct interaction between CypA and any of the EAV nsps (unpublished observations), this suggests that the mechanism by which CypA associates with the viral RTC is sensitive to CsA. In addition, CsA completely blocked EAV RNA synthesis *in vitro*, presumably by targeting the functional association of CypA with preformed RTCs. Therefore, in the most likely model CypA is somehow directly involved in arterivirus RNA synthesis, by interacting with one of the key components of the EAV RTC. In this context, viral the helicase (nsp10) is of particular interest since it plays a role in RNA synthesis and contains several predicted CypA binding domains (**Chapter 3**). Previously, for SARS-CoV it has been shown that CypA interacts with the RNA-binding N protein [312]. In the case of EAV, it is unlikely that an interaction between CypA and N is crucial for viral RNA synthesis, since the N protein was found to be completely dispensable for both genome replication and sg mRNA synthesis [270].

The different sensitivities to the non-immunosuppressive CsA analogue Debio-064 suggest that mechanistic differences exist between corona- and arteriviruses with respect to the role of Cyps in their replication. EAV and PRRSV replication was inhibited by Debio-064, while SARS-CoV replication was not affected by this compound. This notion is further supported by the fact that CypA or CypB knockdown did not affect SARS-CoV replication, whereas EAV replication was reduced upon CypA depletion. In line with the results of Pfefferle *et al*. [277] and a recent study by Carbajo-Lozoya *et al*. [439], this suggests that the NF-AT pathway is relevant for SARS-CoV replication. In contrast, the Cyps themselves probably directly affect EAV replication. Analysis of mutations acquired by CsA-resistant (escape) mutants of EAV and other nidoviruses might shed light on

the viral proteins involved in functional interactions with CypA and on the mechanistic details of their role in nidovirus replication.

ANTIVIRAL RESEARCH ON CURRENT AND FUTURE EMERGING CORONAVIRUSES

Vaccination was the basis for some of the greatest successes in the history of medicine, including the eradication of smallpox, the near-eradication of polio, and the prevention of considerable morbidity and mortality caused by viral infections. Vaccine development currently is a slow process that is not equipped to combat outbreaks of rapidly emerging viruses (see above), although this may improve in the longer run, for example due better production platforms and vaccine engineering on the basis of viral reverse genetics. An alternative way to combat, or at least slow down, emerging infections is the use of antiviral compounds. Thus far, global research efforts to develop antiviral drugs have resulted in treatments for only a handful of human pathogens, i.e. herpesviruses (herpes simplex virus, varicella zoster virus, Epstein-Barr virus, cytomegalovirus), HIV-1, influenza virus and HCV, the only +RNA virus for which approved antiviral therapy is available.

Antiviral drugs can be divided in two main classes: direct-acting antivirals (DAA) and host-directed antivirals (HDA). DAAs directly target (the function of) viral proteins, during entry, replication or assembly of the virus. In general, due to their specificity, these compounds are very potent viral inhibitors, however the quick rise of viral resistance remains a major issue. Successful antiviral therapies are therefore commonly based on the use of a combination of antiviral drugs, in order to increase the barrier of resistance. To reduce this problem of resistance, the development of HDAs is current receiving more attention, especially for the treatment of HCV. Registered antiviral therapies for HCV are based on targeting the viral protease with Telaprevir or Boceprevir, in combination with stimulation of the antiviral response by treatment with IFNs and using the nucleoside analogue Ribavirin [440-442]. This treatment accomplishes a sustained viral response, which means no detectable virus in the blood for six months after the end of treatment, in up to 75%-85% of the cases [443]. This example illustrates the high potential of combined treatment with DAAs (nucleosides and/or protease inhibitors) and HDAs (IFN). However, adverse effects are common during the current anti-HCV therapy, with half of the patients exhibiting flu-like symptoms and a third experiencing emotional problems. These adverse effects are mainly attributed to the use of IFN. For HCV treatment, the effect of Cyp inhibitors, in combination with Ribavirin and pegylated IFN, is currently being explored in phase III clinical trials [436, 444], although these have been (temporarily) put on hold by the FDA due to safety concerns since a patient died from pancreas failure.

As discussed at the start of this chapter, vaccines that need to be developed at the start are not very suitable against outbreaks that only last for a few months, and a more promising approach is to develop treatments with (broad-spectrum) antiviral drugs. In the case of SARS-CoV infection, the gap of ~10 days between the onset of early symptoms and the peak of viral load provides a good window of opportunity for antiviral treatment [445]. A variety of coronavirus inhibitors has been identified in *in vitro* systems and in cell culture (for an overview, see **Chapter 1**). To evaluate the efficacy of these compounds *in vivo* and to study SARS-CoV pathogenesis, multiple animal models have been developed. Mouse-adapted SARS-CoV strain MA15 [446], transgenic mice expressing the human ACE2 receptor [447], and a SARS-CoV ferret model [190] have all been used to study SARS-CoV infection [399]. However, a well-tolerated and effective treatment of SARS-CoV infection is still not available [187]. It therefore remains very important to continue identifying and optimizing SARS-CoV inhibitors or preferentially pan-coronavirus inhibitors and to keep evaluating those in the available animal models.

Since the start of the MERS outbreak in June 2012, the search for antivirals and the development of convenient animal models have been ongoing in many institutes. So far, virus replication was only observed in rhesus macaques [448] and the lack of (cost-effective) small animal models hampers antiviral studies and is one of the reasons to evaluate already registered drugs for their potential to inhibit MERS-CoV [449]. These drugs have already been evaluated in clinical trials and have well-documented safety and pharmacokinetic profiles in patients. Therefore, using these drugs would greatly accelerate the development of antiviral therapy for MERS infections. We (**Chapter 6**) and others [395, 450-452] have reported that MERS-CoV replication was very sensitive to several FDA approved drugs. Especially IFN treatment strongly inhibited MERS infection, and MERS-CoV was up to 100 times more sensitive to type-I IFNs than SARS-CoV. In cell culture, MERS-CoV replication was inhibited by CsA (**Chapter 6**) and the registered drugs lopinavir and chloroquine (de Wilde *et al*, unpublished observations), which provides a promising starting point for the development of intervention strategies for MERS infections. These antivirals would be desperately needed if the MERS outbreak increases in magnitude and severity and could be a treatment option for other zoonotic coronaviruses that might emerge in the future.