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CHAPTER 8

General discussion

ONE GOAL, MANY SOLUTIONS: MECHANISTIC ALTERNATIVES FOR ARTERIVIRUS RNA SYNTHESIS AND CAPPING

A few words on nidovirus diversity and genome architecture

The order *Nidovirales* with its families *Arteriviridae*, *Coronaviridae* (subfamilies *Coronavirinae* and *Torovirinae*), *Mesoniviridae*, and *Roniviridae* comprises members that are genetically more distant from each other than the most diverged organisms of the Tree of Life (1-6). It may thus be asked, what defines a nidovirus and what unites members of the order. On the protein level three enzymatic subunits, the chymotrypsin-like main protease $3CL^{pro}$, the RNA-dependent RNA polymerase (RdRp), and the superfamily (SF) 1 helicase, are the most conserved (1). However, none of these enzymatic domains can be considered nidovirus-specific as they are also common in many other positive-stranded (+) RNA viruses. Instead, bioinformatics studies identified the N-terminal domains of the proteins including also the RdRp or helicase domains as genetic markers for the order (chapter 5 and (5)). On a higher level, nidoviruses share a unique genome organization comprising a conserved array of features, encoded in the two 5′ replicase open reading frames (ORFs), and multiple 3′ ORFs that are translated from subgenomic (sg) mRNAs. In the 5′ to 3′ direction, this array includes the $3CL^{pro}$ flanked by two transmembrane proteins, a ribosomal frameshift site (RFS), the RdRp, and the helicase. The positioning of the helicase subunit downstream of the RdRp is not observed for any other group of (+) RNA viruses, and the implications of this organization have remained elusive so far. However, it is assumed that the particular arrangement of the array reflects strong constraints due to certain essential and universal requirements of the nidovirus replication cycle, which are poorly understood (5). Next to these core attributes, additional less conserved domains may be integrated into the replicase of specific members or subgroups of the order (see Figure 1 of chapter 6, p. 199). These additional domains, as well as a further expansion of the size of conserved proteins, were the major contributors to the lineage-specific increase in genome length of intermediate (*Mesoniviridae*) and large (*Coronaviridae*, *Roniviridae*) nidoviruses compared to that of the smaller arteriviruses (7). The profound divergence of nidovirus families on the protein level must have functional implications and calls for caution when attempting to generalize our limited biochemical knowledge on nidoviruses, which is based on studies of only a few corona- and arteriviruses. In this context, the following paragraphs discuss alternative interpretations of the arterivirus data presented in this thesis and elsewhere and formulate plausible hypotheses, which are worth pursuing in future studies on the molecular details of arterivirus replication.

Initiation of RNA synthesis

The copying of genetic information with the intent to produce progeny and blueprints for protein production is one of the few characteristics viruses and living organisms have in common. It is therefore not surprising that the "cupped right-hand" structure of canonical polymerases and their mechanisms are heavily conserved, even among otherwise genetically distant viruses and organisms (8;9). Nevertheless, some variation exists in the mechanisms used to initiate nucleic acid synthesis. In general two types of initiation can be distinguished: primer-dependent and primer-independent (also called *de novo*). Polymerases capable of the latter start nucleic acid synthesis by joining two NTPs, either independently or in association with a template but not necessarily with its 3' end. This dinucleotide then gets elongated in the 5'-3' direction in a strictly template-dependent manner. In contrast, primer-dependent polymerases rely on another enzyme to generate this starting dinucleotide (9). Which type of initiation a polymerase utilizes has been associated with the presence (primer-dependent) or absence (*de novo*) of the conserved so-called G motif in the enzyme (10). Particularly, this motif was found in the putative RdRps of the *Arteriviridae* and *Coronaviridae* (11;12), and of the other two nidovirus families (Gorbalenya, personal communication). In the RdRp of severe acute respiratory syndrome coronavirus (SARS-CoV), designated nsp12, the presence of this motif was correlated with primer-dependent RdRp activity (13). These observations thus provided a rationale for the earlier described, but still debated, non-processive *de novo* RdRp activity of a smaller coronavirus protein, nsp8, encoded upstream of the RFS and lacking canonical polymerase motifs (14-16), which was proposed to generate the primers for the nsp12-based RdRp. In contrast to the situation for coronavirus nsp12, the G motif was found to be incomplete in the arterivirus equine arteritis virus (EAV) RdRp designated nsp9 (12). Additionally, the protein with the proposed "accessory RdRp" activity was found to be conserved in corona- and toroviruses, but its presence in other nidoviruses is uncertain due to very low sequence similarity in the corresponding part of the replicase protein (Gorbalenya, personal communication). It may therefore be speculated that, contrary to its coronavirus homolog, the RdRp encoded downstream of the RFS in arteriviruses is itself a *de novo*-initiating polymerase. This hypothesis was seemingly confirmed in *in vitro* assays using recombinant EAV nsp9 (12). Yet, this finding could not be extended to activity on natural EAV RNA templates and could furthermore not be reproduced in the present study (chapter 4).

Interestingly, EAV nsp9 was shown to possess a second enzymatic activity (chapter 5), which may be part of an alternative priming mechanism that is independent of the recruitment of a second cognate RdRp acting as a primase. This mechanism, which is exclusively employed by viral polymerases, is characterized by the formation of short,

protein-linked polynucleotides that are subsequently positioned at the template's 3′end to allow elongation by the polymerase. The enzyme activity catalyzing this reaction is, where known, exerted by the polymerase itself with the help of a substrate protein that is typically named VPg (viral protein genome-linked) and ranges in size from 20 to more than 200 amino acids (17;18). So far this type of "protein priming" was described for double-stranded (ds) DNA viruses of the *Hepadnaviridae (19)*, dsRNA viruses of the *Birnaviridae (20)*, and (+) RNA viruses of the *Picornavirales* (21-25), *Caliciviridae (26)*, *Potyviridae* (27;28), *Permutotetraviridae* (29), and *Astroviridae* (30). Intriguingly, despite having genomes of only half the size of those of arteriviruses, the latter share the basic genome organization of nidoviruses including the 3CL^{pro}-RFS-RdRp array and expression of structural proteins from a 3′-coterminal sg mRNA (Figure 1). It was therefore speculated that an early nidovirus ancestor might have resembled the contemporary astroviruses (5). Although this genetic similarity is no guarantee that also mechanistic details of viral replication correspond, it is tempting to speculate that the mode of initiation of RNA synthesis might only have been altered upon acquisition of a second RdRp in the course of the genome expansion of the large (and intermediate) nidoviruses.

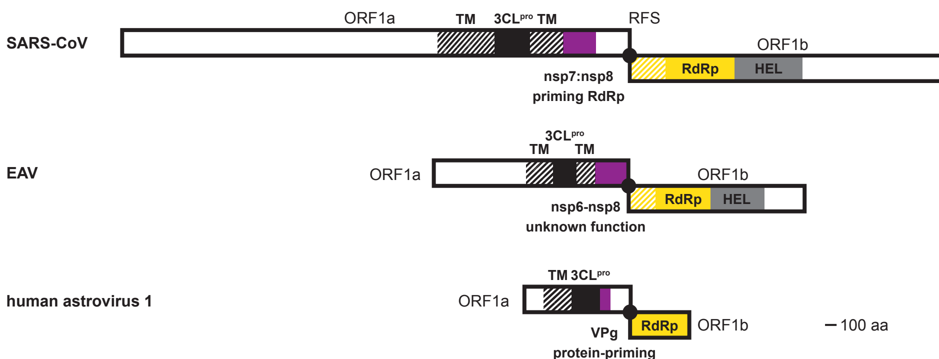

Figure 1: Replicase organization of nidoviruses and human astrovirus. Protein domains belonging to the conserved functional array of nidoviruses and their counterparts in astrovirus are indicated. Proteins that are known or hypothesized to take part in the initiation of RNA synthesis are depicted in purple. TM, transmembrane domain; 3CL^{pro}, 3C-like protease; black dot and RFS, ribosomal frameshift site; RdRp, RNA-dependent RNA polymerase; HEL, helicase/RNA triphosphatase (adapted from (5)).

In the first step of protein-priming a nucleotide monophosphate is covalently attached to the substrate protein under release of pyrophosphate, a reaction classified as nucleotidylation. In most cases this substrate is not part of the polymerase subunit itself, but the extent of auto-nucleotidylation may vary in response to reaction conditions, as it was described for the RdRp of poliovirus (*Picornavirales*) (23). Currently, the only known notable exceptions to this general trend are proteins of the hepadnavirus hepatitis B virus and birnaviruses, which simultaneously serve as enzyme and substrate (19;20).

Furthermore, for infectious bursa disease virus (*Birnaviridae*) VP1, which contains the RdRp domain, it was shown that auto-nucleotidylation at the site located upstream of the RdRp domain does not depend on the conserved polymerase active site (20). In contrast, such a dependence was observed for VP1 of infectious pancreatic necrosis virus, another distantly related birnavirus (31). That study demonstrated a template-independent auto-guanylylation activity that modified a serine residue conserved in birnaviruses. Interestingly, only the fraction of VP1 molecules that served as primers was guanylylated while other, non-modified RdRp molecules served in elongation (32). This mechanism may also be conserved in the (+) RNA *Permutotetraviridae* (29).

In these respects nucleotidylation of EAV nsp9 (chapter 5) may behave quite similar to that seen in birnaviruses. However, a rough estimation indicated that only a very small fraction (<1%) of nsp9 proteins was labeled with UMP or GMP – not taking into account the potential presence of inactive nsp9 molecules, which may be numerous given the instability of this recombinant protein. It may thus be questionable if a birnavirus-like division between priming and elongating protein fractions can be envisioned for EAV nsp9. In addition, we noticed a tendency to transfer this label to other EAV proteins but also unrelated polypeptides. Yet, as we were so far unable to identify a protein that serves as the preferred acceptor for UMP or GMP, we currently consider this transfer activity an artifact of the *in vitro* assay, maybe due to the general instability of the phosphoamide bond that is formed between the nucleotides and nsp9. Nevertheless, the low nucleotidylation efficiency and nonspecific transfer may be indicators of suboptimal reaction conditions, especially a lack of co-factors that may enhance nsp9 activity or serve themselves as VPg. Given the numerous replicase subunits and long-lived cleavage intermediates without an assigned function in arteriviruses (33), it can only be speculated which subunits might fulfill such a function. Strikingly, all currently known co-factors of RNA-processing enzymes in nidoviruses, specifically coronavirus nsp7, 8, and 10 (15;16;34), derive from the region between the transmembrane domain downstream of the 3CL^{pro} and the RFS. Could an arterivirus VPg also be derived from that region, which comprises nsp6 to nsp8? Interestingly, arterivirus nsp6, a conserved 11 to 22 amino acid peptide, is known to be part of a number of uncharacterized cleavage intermediates that are subject to alternative processing pathways in EAV (33). Next to the fully cleaved nsp6, one of these nsp6-containing intermediates may be considered as initial nucleotidylation substrate, whose regulated cleavage may be a convenient way to reduce affinity for and thus prevent retention of the polymerase at the RNA 5′ end once initiation has occurred. Finally, to invoke again the above mentioned similarity to astroviruses, it remains to be noted that the VPg of this virus family is indeed located between 3CL^{pro} and the RFS (30) (Figure 1).

After auto-nucleotidylation of the RdRp-containing protein or its nucleotidylation of the VPg, the first nucleotide is extended by one or more additional nucleotides to generate a sufficient platform for annealing to the template strand. Thus, if the specificity of the nucleotidylation reaction *in vitro* faithfully reflects that *in vivo*, it must match the conservation of the 5′ ends of genome and/or antigenome. In the case of the EAV Bucyrus strain, which was the source of the nsp9 characterized in chapter 5, this sequence is GCU for the genome and GGU for the antigenome. Due to the unique replication mechanism of nidoviruses, all sg mRNAs and subgenome-size negative-stranded RNAs would contain identical 5′ ends as the genome or antigenome, respectively (5;35). Although no evidence for an elongation of the first nucleotide was obtained in our experiments (chapter 5), it is noteworthy that for both RNA polarities nucleotidylation using GTP as substrate would be consistent with the observed nucleotide sequences. Intriguingly, bovine coronavirus was reported to encode a short poly uridine (poly(U)) tract at the antigenome's 5′ end, which is thought to serve as template for poly(A) tailing of all mRNAs (36). The antigenome sequence has not been characterized for any arterivirus, but if it includes poly(U) at the 5′ end, it could explain the dual specificity of nsp9 nucleotidylation. In contrast, neither the presence of this poly(U) tract nor the sequence of the genomic 5′ end can be easily reconciled with *de novo* synthesis, which is assumed to generally require two purines as start nucleotides based on the biochemical characterization of a number of polymerases (9). To accommodate *de novo* synthesis, arteriviruses would thus require additional editing of one or both 5′ ends. In contrast to large nidoviruses, encoding dedicated enzyme domains for this function may represent a significant burden to arteriviruses.

Despite these arguments for protein-primed RNA synthesis in EAV, there is one complication with this hypothesis since the chemical nature of the protein-nucleotide bond in the previously characterized VPgs does not match that between nsp9 and GMP/UMP. In all viruses with protein-primed replication that were investigated in detail the protein-nucleotide bond was established with the help of either a tyrosine or serine, in other words via a hydroxyl moiety (17;18;20;23). In EAV nsp9 the bond to the GMP/UMP is formed via the side chain amino group of a lysine or less likely histidine (chapter 5), and thus the situation would be more similar to that observed in nucleotidylating enzymes involved in nucleic acid ligation or mRNA capping (both discussed in detail in chapter 5) (37-39). This difference could have profound functional implications, because phosphoesters are known to be chemically more stable under physiological conditions than phosphoamides (40). However, arteriviruses may have evolved to have a labile bond between nsp9 and the nucleotide to allow the subsequent nucleotide transfer to a genuine VPg. This two-stage mechanism would provide an additional level of control and plasticity, with nsp9 being the designated carrier of the transferrable nucleoside

monophosphate. Furthermore, the instability and therefore transient nature of the bond would only impact EAV replication if the protein modification at the 5′ end would serve functions beyond initiation of RNA synthesis that are required throughout the entire lifetime of the RNA, for example nuclease protection or translation initiation. The latter function is fulfilled by calicivirus VPgs, which substitute for 5′ mRNA nucleotide cap structures and bind directly to the translation initiation factor eIF4E (41;42). The fact that the putative bond between EAV RNA and a terminal protein might be unstable, may thus merely indicate that this virus utilizes a secondary mechanism to modify its mRNAs with regular cap structures after the transiently bound VPg has been removed.

mRNA 5′-terminal modifications

Besides protein-priming, nucleotidylation could be implicated in the formation of the cap structure of mRNAs. Nidovirus mRNAs are thought to contain a type 1 cap structure (cap-1) ($^{m}GpppN_{m}$) at their 5′ end that enables translation in the absence of special RNA secondary structure elements as internal ribosome entry sites (IRESs) or 3′ cap-independent translation enhancers (CITEs). However, some experimental evidence supporting that assumption has only been obtained for equine torovirus (43), the coronavirus mouse hepatitis virus (MHV) (44;45), and the arterivirus simian hemorrhagic fever virus (46). As the question regarding the 5′ modification was not addressed for either roniviruses or mesoniviruses and a contradicting report exists for another arterivirus (47), it is far from proven that the presence of a cap-1 is a universal feature of the order. Additionally, there is considerable uncertainty about nidoviruses universally encoding a set of specific enzymes that were shown to be required to produce the cap-1 structure in better characterized (+) RNA viruses (Figure 2). While two of the enzymatic activities that are essential for the four-step synthesis of the cap-1 may reside in the nidovirus-wide conserved N-terminal domain of the RdRp subunit (chapter 5) and the helicase subunit (chapter 3), the two methyltransferase domains were so far only identified in coronaviruses, roniviruses, and mesoniviruses (6;48-52) but not in arteriviruses (7). Yet, methylation of the cap serves, on the one hand, translation initiation (N7-methylation) via the recruitment of eIF4E and, on the other hand, immune evasion (2′-O-methylation) (53-55). Therefore in theory, at least N7-methylation should be an essential step in the transcription of nidoviral mRNAs if it follows mechanisms established for other viruses. In chapter 6 we investigated whether the arterivirus-specific protein nsp12 might contain methyltransferase activity but were unsuccessful in verifying this hypothesis, potentially due to purely technical reasons. Thus, it remains an open question how arteriviruses would achieve the complete synthesis of a cap-1.

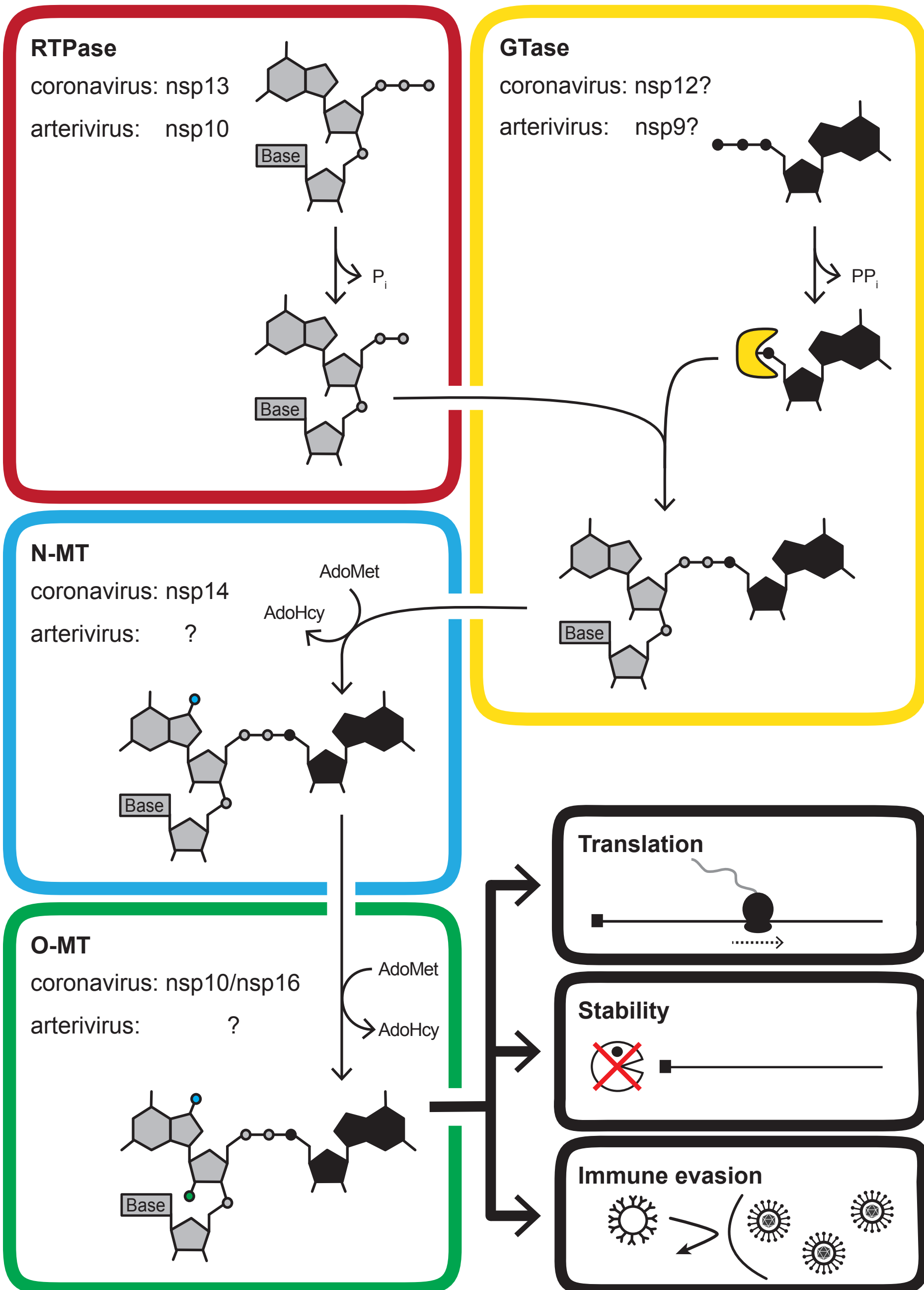

Figure 2: Conventional mRNA capping mechanism, nidovirus proteins (putatively) involved, and functional roles of the cap. RTPase, RNA 5'-triphosphatase; GTase, guanylyltransferase; N-MT, N7-methyltransferase; O-MT, 2'-O-methyltransferase; AdoMet, S-adenosylmethionine; AdoHcy, S-adenosylhomocysteine.

Besides nsp12 supplying the N7-methyltransferase activity in arteriviruses, several other options could be considered. First, the recruitment of cellular enzymes, which are located in the nucleus (56), seems to be a possibility potentially through the involvement of dedicated viral proteins that shuttle to the nucleus. Another option is snatching a cap structure from cellular mRNAs. For this purpose, several groups of negative-stranded (-) RNA viruses that employ this mechanism have evolved specific cap-binding and endoribonuclease domains (57-60). Also arteriviruses are known to encode an endoribonuclease that is associated with nsp11 (61). However, upon analysis of genomes and mRNAs of established cap-snatching viruses of the *Orthomyxoviridae*, *Bunyaviridae*, and *Arenaviridae*, it became evident that all of these viruses harbor at least one (arenaviruses) and up to 17 (bunyaviruses) nucleotides at their mRNA 5′ ends that are variable and not virus-encoded (62;63). Since such host-derived sequences have not been discovered in the extensive study of arterivirus 5′ untranslated regions (UTRs) (47;64-66), the utilization of an analogous cap-snatching mechanism by arteriviruses seems very unlikely.

Noteworthy, a variant of cap-snatching in which only the terminal ^{m}GMP moiety is removed from cellular substrates is employed by the dsRNA viruses of the *Totiviridae (67)*. Still, if this mechanism, or a variation thereof, is considered for arteriviruses, other incompatibilities between its characteristics and our knowledge about arteriviruses become apparent. Particularly, cleavage within the peculiar 5′-5′ linkage of the cap is usually not catalyzed by ribonucleases that are able to cleave regular 5′-3′ bonds. Although the physiological substrate for nsp11 has not been established yet, its demonstrated *in vitro* specificity for pyrimidine-containing single- and double-stranded RNAs makes it unlikely that this unusual bond would fall within the enzyme's substrate range (61). Finally, one of the arterivirus proteins may specifically recognize the 5′ end of arterivirus mRNAs and facilitate translation initiation without the need for eIF4E involvement and hence potentially independent of N7-methylation.

To conclude, the remaining significant gaps in our understanding of arterivirus RNA synthesis and 5′ modification(s) currently leave space to formulate a number of parallel hypotheses. Given that arteriviruses are even considerably well characterized compared to all nidovirus families other than coronaviruses, a significantly bigger effort regarding biochemical and structural studies is required to establish universal and lineage-specific mechanisms in different families of the *Nidovirales*.

THE IMPORTANCE OF HOST HELICASES FOR NIDOVIRUS REPLICATION

In chapters 2 and 3 potential functions of the conserved UPF1-like nidovirus helicase have been discussed. Reflecting the enzyme's versatile nature, those include roles during replication, transcription, RNA modification and processing, as well as virion biogenesis, which place this protein at the very center of the nidoviral replication cycle. Despite these prominent roles in nidoviruses and other (+) RNA viruses with a genome larger than 7 kilobases, helicases are not encoded by retroviruses or (-) RNA viruses, which are, for the most part, considerably larger than 7 kilobases (68). This may be explained by significant differences in their respective replication cycles compared to (+) RNA viruses, which, on the one hand, may have rendered those viruses helicase-independent or, on the other hand, may have enabled them to recruit cellular helicases. A case in point is the retrovirus human immunodeficiency virus, whose proteins or RNA elements interact with at least seven host RNA helicases – MOV10 (Upf1-like, SF1), DDX1, DDX3, DDX24 (all DEAD-box family, SF2), DHX9, DHX30 (both DEAH-box family, SF2), and RH116 (DExH-box family, SF2) – to promote, amongst others, transcription initiation, translation, and virion infectivity (reviewed in (69)). Furthermore, recently DDX21 was implicated in the temporal regulation of influenza A virus gene expression (70).

Interestingly, utilization of host helicases for specific functions during their replication cycle is not exclusively a feature of viruses that do not encode their own helicase. For example, also the helicase-encoding bovine viral diarrhea virus (BVDV), hepatitis C virus (both *Flaviviridae*), and foot-and-mouth disease virus (FMDV, *Picornaviridae*) were found to depend on DHX9 for genome replication. While the exact role of this protein in BVDV infection remains unknown (71), the latter two viruses likely require this host factor for circularization of viral RNAs, as the protein was shown to bind to the 5' and 3' UTRs of the viral genome (72;73). Furthermore, in FMDV the same protein co-immunoprecipitated with the viral SF3 helicase 2C and nonstructural protein 3A (72). Together these results may indicate that cellular helicases are not only required to exert their enzymatic activities but may also serve as scaffolds for the assembly of multimeric protein-RNA complexes via their accessory domains.

Although the number of RNA viruses with proven dependence on host cell helicases is currently small, it can be expected that more and more of these host factors will be identified due to the rising popularity of large-scale siRNA, yeast two-hybrid, and proteomics screens. For example, these approaches recently led to the discovery of an interaction between DDX1 and two coronavirus proteins, nsp14 and the nucleocapsid protein N (74-76). The results of two independent studies addressing these interactions are discussed in more detail below.

Moreover, in recent years a number of RNA helicases other than the well characterized RIG-I and MDA5 have been implicated in cellular antiviral defense mechanisms (77). Curiously, these again include DDX1 and Upf1. Thus, the recruitment of or structural similarity to these cellular helicases possibly serve a dual role in the nidovirus replication cycle. This section is therefore concluded with a short summary on the defense mechanisms mediated by these two proteins.

The cellular helicase DDX1 and nidovirus transcription regulation

As discussed in detail in chapter 3, the nidovirus helicase is one of the few proteins that has been directly implicated in the mechanism of discontinuous negative-strand synthesis that produces the subgenome-length templates for sg mRNA synthesis (78;79). Surprisingly, two studies now also linked the cellular helicase DDX1 to (sg) mRNA synthesis. Originally this host factor, which seems to be involved in 3′ mRNA processing and tRNA splicing in the nucleus of uninfected cells (80;81), was identified in a large-scale yeast two-hybrid screen as an interaction partner of nsp14 of the coronaviruses infectious bronchitis virus (IBV) (76). Further directed investigation by the same group extended this interaction also to SARS-CoV nsp14 and mapped the interaction surface to the N-terminal exoribonuclease domain of this protein. In line with this finding, upon IBV infection of Vero cells the mostly nuclear localization of DDX1 was altered into a cytoplasmic punctuate pattern, similar to that observed for coronavirus replicase proteins. Given this apparent recruitment to replication-transcription complexes, it was not surprising that stable or transient knock-down of DDX1 led to a tenfold decrease of virus peak titers. Interestingly, when examining the levels of N and S protein expression – produced from the shortest and longest sg mRNAs, respectively, in IBV – the amount of S protein appeared to be significantly reduced upon DDX1 knock-down while the amount of N protein was not affected. This finding correlated with the preferential decrease of the transcription level of longer mRNAs (subgenomic and genomic) compared to shorter ones, which was also observed. Since this effect had the same relative magnitude for RNAs of both polarities, it was speculated that DDX1 might be involved in the regulation of the relative abundance of individual negative-stranded RNAs (76). Notwithstanding the fact that this hypothesis was devised to explain the features of coronavirus replication, this host factor may thus act on a different level or by a different mechanism than the endogenous nidoviral helicase, whose mutation may impair the synthesis of all sg mRNAs uniformly and selectively relative to genomic RNA production in EAV (79). Whether or not this regulatory mechanism actually involves the proven interaction with IBV nsp14 was not established. However, it was noted that continued passaging of IBV in DDX1 knock-down cells did neither induce mutations in nsp14 nor had any impact on viral fitness, which would be expected if nsp14 proofreading would be affected by

DDX1. Based on these results, it seems more likely that the nsp14-DDX1 interaction plays a different, as yet unidentified, role in the coronavirus replication cycle (76).

A few years after this study, the same host helicase was identified as interaction partner of the IBV and MHV N proteins both in the absence and presence of cellular RNA (74;75). As for IBV earlier, also the MHV study demonstrated the selective reduction of longer RNA species upon DDX1 knock-down. Additionally, subsequent ectopic over-expression of knock-down resistant, functional DDX1 but not of a helicase active site mutant led to a reversal of this effect, establishing its dependence on helicase activity. In contrast to the study detailed above, it was furthermore demonstrated that abolishing the interaction between N and DDX1 by preventing phosphorylation of N at serine 197 had the same impact on RNA abundance as DDX1 knock-down. It was thus concluded that complex formation between these two proteins may promote read-through at transcription regulatory sequences during discontinuous negative-stranded RNA synthesis (75). However, this would imply that abolishing complex formation should not only specifically diminish the quantity of longer RNA species but at the same time also increase that of shorter RNAs if no other limiting factor plays a role. Neither of the two studies reported such an outcome (75;76). Instead total RNA amounts were reduced while that of short sg mRNAs remained largely constant. The most obvious alternative explanation for this pattern would therefore be a direct stimulation of RdRp processivity by the N:DDX1 complex. This, however, is unlikely to be true as the synthesis of genomic RNA, which is more than three times as long as the longest sg mRNA, was affected to the same extent as that of this latter sg mRNA. Next to a direct involvement in RNA synthesis, the reported data would also be consistent with a role for the N:DDX1 complex in a selective stabilization of certain negative-stranded RNAs before mRNA synthesis commences. In order to elucidate the exact role of DDX1, a deeper understanding of the nidovirus replication mechanism and potential downstream regulatory pathways influencing the stability of the negative-stranded subgenome-length RNAs would be required. Yet, this appears to become an ever more daunting task with every additional protein, viral or cellular, that is implicated in the nidovirus replication cycle.

Cellular helicases and antiviral defense

In the course of evolution eukaryotic cells have developed an intricate defense system to counteract infections by bacterial, eukaryotic, and viral pathogens. A central role in one arm of this system, the innate immune system, is played by conserved pattern-recognition receptors that recognize a certain signature molecule of defined groups of pathogens. In the case of RNA viruses this recognition largely depends on the sensing of viral nucleic acids inside the cell. The proteins responsible for detection are the endo-

somal Toll-like receptors 3, 7, and 8, as well as cytosolic NOD-like and RIG-I-like receptors. The latter group is comprised of the three DExH-box SF2 RNA helicases RIG-I, MDA5, and LGP2, of which MDA5 was shown to sense MHV RNA (82). Once these receptors bind their ligand, a complex cascade of downstream effectors is activated, eventually leading to the transcription of genes involved in inflammatory response and cross-talk with the adaptive immune system. Most notably is the production of type I interferons, which in turn indirectly regulate protein synthesis, cell growth, and apoptosis (reviewed in (83)).

As would be expected, during an extensive period of co-evolution with their hosts, viruses have developed a variety of means to avoid, inhibit, or redirect essential factors of the innate immune system. For instance, nidoviruses, as all other characterized (+) RNA viruses infecting eukaryotes, are known to induce extensive membrane modifications inside the host cell (84). Since these are thought to be the site of viral RNA synthesis, they may serve to hide viral nucleic acids, in particular the highly immunogenic double-stranded replication intermediates, from cytosolic sensors. Additional avoidance strategies that could be employed by at least a subset of nidoviruses are the disguising of viral RNAs by attaching the typical eukaryotic double-methylated cap structure to mRNA 5′ ends or the degradation of an excess of viral RNAs by either of the two viral ribonucleases (85). Furthermore, the nsp1α, nsp1β, and nsp4 proteases of the arterivirus porcine reproductive and respiratory syndrome virus (PRRSV), as well as the PLP2 protease domains of EAV and PRRSV nsp2 have been implicated in the inhibition of immune signaling although interestingly not in all cases through their proteolytic activity (86). Also coronavirus nsp1 and nsp3 appear to be engaged in immune suppression (87-89). It remains to be seen whether similar strategies have also evolved in the other nidovirus (sub)families. Yet, these examples show that a significant number of proteins may be dedicated to counteract the host’s defense mechanisms and to shape a more beneficial environment for virus replication. Thus, given the extensive divergence between individual members of the order, it is currently difficult to estimate how large the repertoire of nidovirus evasion strategies really is.

DEAD/H-box helicases and RNA detection

Interestingly, a number of SF2 RNA helicases besides RIG-1 and MDA-5 were identified as additional players in virus sensing and immune signaling in recent years (77). One of those is the above mentioned DDX1, which was shown to bind to poly(I:C) RNA and may recognize any RNA species. Binding of DDX1 to a substrate promotes the complex formation with two other helicases, DDX21 and DHX36. Both of these helicases can subsequently interact with the innate immunity signaling protein TRIF and thereby induce an inflammatory response. The importance of this pathway was demonstrated in studies

on influenza virus and reovirus, in which interferon production was reduced once any of the three helicases was knocked-down (90). Additionally, DDX1 is able to directly bind to the RelA subunit of the pro-inflammatory transcription factor NF-κB, thereby stimulating transcription activation by this factor (91). Intriguingly, next to DDX1, also DDX21 and DHX36 were identified as putative interaction partners of the IBV N protein by co-immunoprecipitation (74). The latter interaction was also found for PRRSV (92).

Further RNA helicases that are involved in immunity and that were identified as binding partners of N in IBV and PRRSV are DDX3 and DHX9 (74;77;92). The former, which also serves in translation regulation especially of mRNAs with complex 5′ UTRs (93), acts as a sensor for dsRNA. However, unlike signaling by the DDX1 complex, the pathway for this helicase is identical to that of RIG-I and involves the downstream effector MAVS (94). Similarly, also the transcriptional regulator DHX9 was shown to interact with MAVS upon encountering dsRNA (95). It was therefore speculated that both DDX3 and DHX9 may be of particular importance early in infection when the RIG-I concentration is still low. Whether any of these cellular helicases actually plays a role in nidovirus sensing, immune evasion, or replication remains to be seen. Nevertheless, it is an interesting possibility that the interaction of these host proteins with N might interfere with their immune signaling responsibilities.

Upf1 and NMD-mediated defense

In eukaryotic cells the relative abundance of mRNAs is heavily regulated. One of the involved mechanisms, which controls the quantities of up to 10% of all transcribed mRNAs, is termed nonsense-mediated decay (NMD). Besides this function, the same mechanism also controls ribosome release from, as well as translation repression and – ultimately – decay of aberrant transcripts with, for example, premature stop codons that may arise due to wrong or incomplete splicing and nonsense or frameshift mutations. Although NMD has been studied extensively in different species, neither its RNA or protein triggers nor the exact sequence of events involved in this process are well understood. Notwithstanding this uncertainty, the SF1 helicase Upf1 and the poly(A) binding protein (PABP) – or more precisely the competition between these proteins – appear to be of special importance. During translation of wild-type host mRNAs, PABP is bound sufficiently close to the terminating ribosome to establish an interaction with the termination factor eRF3, which in turn stimulates termination and triggers ribosomal release. Conversely, if the distance between eRF3 and PABP is artificially elongated by, for instance, the introduction of an upstream stop codon or the presence of a second downstream ORF, termination becomes less efficient. In this situation Upf1 is able to compete with PABP for eRF3, triggering the formation of a

larger protein complex, which marks this mRNA for decay (reviewed in (96;97)). While cellular mRNAs have evolved to contain the correct spacing – on average 700 to 800 nucleotides in humans – between stop codon and poly(A) tail (98), some viral RNAs comprising multiple ORFs and elongated 3′ UTRs may be particularly vulnerable to this quality control mechanism. This assumption was recently confirmed with the help of the (+) RNA viruses potato virus X (*Alphaflexiviridae*) and Semliki Forest virus (SFV) (*Togaviridae*) (99;100), which both utilize 3′ co-terminal sg mRNAs. For the plant virus a mutation within the Upf1-gene was shown to lead to an increase in the amount of sg mRNAs with long 3′ UTRs compared to the wild-type situation. At the same time, the abundance of the shortest sg mRNA comprising only a very short 3′ UTR was unaffected (99). Similarly, knock-down of Upf1 extended the half-life of the SFV genomic RNA in HeLa cells from 63 min to 89 min. Consequently, viral titers increased by threefold. This effect could be reversed by ectopic expression of a knock-down resistant functional Upf1 but not an helicase active site mutant. Surprisingly, shortening of the ~4000 nucleotide 3′ UTR of the SFV genomic RNA to 62 nucleotides did not abolish Upf1-mediated decay, a finding which stands in sharp contrast to the observations for the plant virus (100). Even more puzzling is the proven independence of the antiviral mechanism from 5′ cap but especially 3′ poly(A) tail (99). These observations essentially argue for a non-canonical NMD mechanism being involved during antiviral defense. Since two (-) RNA viruses, respiratory syncytial virus (*Paramyxoviridae*) and Uukuniemi virus (*Bunyaviridae*) that strictly encode monocistronic mRNAs, were not affected by Upf1 knock-down (100), the polycistronic nature of mRNAs appears to be the common denominator of this pathway at the moment. Future research may reveal whether different RNA features emerge as triggering factors.

As already speculated for cellular NMD, specific RNA sequences or secondary structures might have evolved to recruit NMD antagonists or inhibit NMD in other ways (96). Given this context, if this defense mechanism actually plays a role for at least a subset of viruses, it would be expected that these viruses employ certain mechanisms to counteract Upf1-mediated decay (101). In view of this assumption, it is intriguing that all nidoviruses encode a helicase that structurally resembles Upf1 (chapters 2 and 3). Although a direct role of this viral protein in RNA quality control might be a preferable explanation for its fixation in the ancestral nidovirus genome, one could envision a secondary function of the nidovirus helicase in counteracting cellular defenses. However, due to its presumed multiple roles during viral replication, confirming this immune evasion strategy will not be an easy task. In addition, Upf1 is known to be a central player in a number of pathways that involve the manipulation of nucleic acids and each employ different protein complexes (96;97). Further research in this area should therefore initially focus on establishing and rigorously validating host interactions with the nidovirus helicase.

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