

# Host Factors in Coronavirus Replication

Adriaan H. de Wilde, Eric J. Snijder, Marjolein Kikkert  
and Martijn J. van Hemert

**Abstract** Coronaviruses are pathogens with a serious impact on human and animal health. They mostly cause enteric or respiratory disease, which can be severe and life threatening, e.g., in the case of the zoonotic coronaviruses causing severe acute respiratory syndrome (SARS) and Middle East Respiratory Syndrome (MERS) in humans. Despite the economic and societal impact of such coronavirus infections, and the likelihood of future outbreaks of additional pathogenic coronaviruses, our options to prevent or treat coronavirus infections remain very limited. This highlights the importance of advancing our knowledge on the replication of these viruses and their interactions with the host. Compared to other +RNA viruses, coronaviruses have an exceptionally large genome and employ a complex genome expression strategy. Next to a role in basic virus replication or virus assembly, many of the coronavirus proteins expressed in the infected cell contribute to the coronavirus-host interplay. For example, by interacting with the host cell to create an optimal environment for coronavirus replication, by altering host gene expression or by counteracting the host's antiviral defenses. These coronavirus-host interactions are key to viral pathogenesis and will ultimately determine the outcome of infection. Due to the complexity of the coronavirus proteome and replication cycle, our knowledge of host factors involved in coronavirus replication is still in an early stage compared to what is known for some other +RNA viruses. This review summarizes our current understanding of coronavirus-host interactions at the level of the infected cell, with special attention for the assembly and function of the viral RNA-synthesising machinery and the evasion of cellular innate immune responses.

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A.H. de Wilde · E.J. Snijder · M. Kikkert · M.J. van Hemert (✉)  
Molecular Virology Laboratory, Department of Medical Microbiology,  
Leiden University Medical Center, Leiden, The Netherlands  
e-mail: m.j.van\_hemert@lumc.nl

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## 1 Introduction

Around the end of 2002, an outbreak of a previously unknown severe acute respiratory syndrome (SARS) started in South East China and Hong Kong. Accelerated by air travel, the disease rapidly spread to several parts of the world and displayed pandemic potential. SARS-coronavirus (SARS-CoV) was identified as the causative agent of this zoonotic infection (Drosten et al. 2003; Ksiazek et al. 2003; Kuiken et al. 2003; Peiris et al. 2003), which resulted in >8000 laboratory-confirmed cases and 774 associated deaths worldwide (WHO 2004). Although in terms of death toll not comparable to influenza, HIV or HCV, the 2003 SARS-CoV outbreak caused worldwide public concern and seriously affected the global economy [estimated losses \$30–100 billion; (Keogh-Brown and Smith 2008)]. SARS-CoV initially causes lower respiratory tract disease, which can lead to a progressive and potentially lethal atypical pneumonia with clinical symptoms that include fever, malaise, lymphopenia, and in some cases also diarrhea. Two years after the outbreak, horseshoe bats were identified as the likely reservoir of the SARS virus, whereas civet cats probably have served as intermediate host during the zoonotic transfer to humans (Lau et al. 2005; Li et al. 2005b). Adaptation to the human host required a small number of mutations in the receptor-binding domain of the SARS-CoV spike (S) protein, which mediates cell binding and entry (Li et al. 2005c) (see Chap. 2). There is increasing evidence that SARS-like coronaviruses continue to circulate in bats and that these may have the potential to readily cross the species barrier and emerge as human pathogens (Ge et al. 2013; Menachery et al. 2015). Such zoonotic scenarios therefore remain a serious public health concern.

Almost a decade after the SARS-CoV outbreak, the next zoonotic coronavirus emerged: Middle East Respiratory Syndrome coronavirus (MERS-CoV) (de Groot et al. 2013). The virus was first isolated in June 2012 from a 60-year-old Saudi Arabian male who died from acute respiratory distress syndrome (ARDS) and multiple organ failure, including renal failure (Zaki et al. 2012; van Boheemen et al.

2012). Also MERS-CoV can cause a lower respiratory tract infection with symptoms that include coughing and high fever. By the end of 2016, more than 1850 laboratory-confirmed MERS-CoV cases had been recorded, with a mortality rate of about 35% (WHO 2016). MERS-CoV is assumed to be transmitted to humans from camels and serological studies in the latter animals revealed that they have harbored MERS-CoV or MERS-CoV-like viruses for decades (Muller et al. 2014).

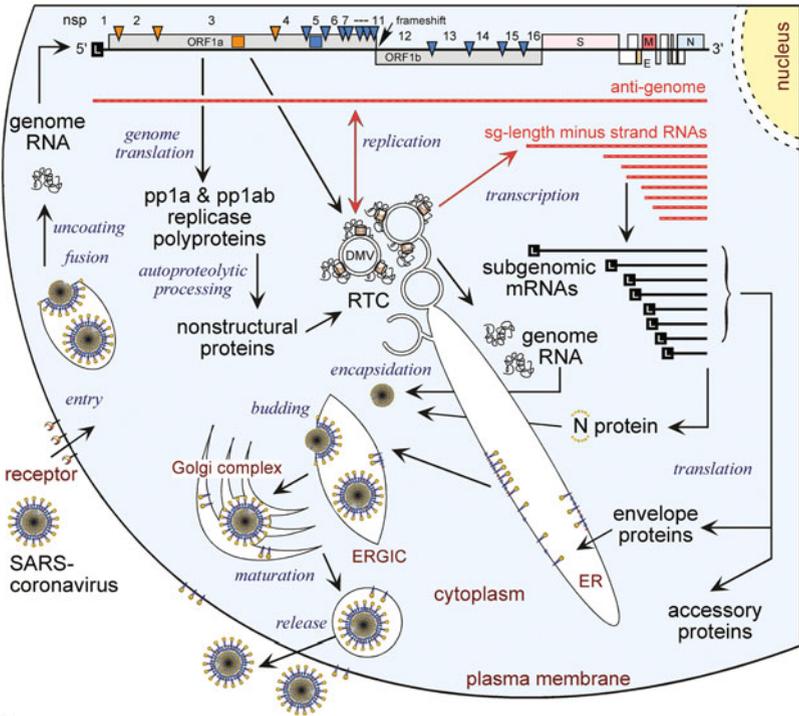
Besides the zoonotic SARS- and MERS-CoVs, the coronavirus family includes four ‘established’ human coronaviruses (HCoVs), of which HCoV-OC43 and -229E have already been known since the 1960s. These two viruses cause mild respiratory disease and, after rhinoviruses, are a leading cause of common colds (10–30% of the cases) (van der Hoek 2007; McIntosh et al. 1967; Hamre and Procknow 1966). More recently, following intensified screening for coronaviruses, two additional HCoVs were discovered, HCoV-NL63 (van der Hoek et al. 2004) and HCoV-HKU1 (Woo et al. 2005). Interestingly, recent findings suggest that also HCoV-NL63, -229E, and -OC43 originate from zoonotic transfer from bats (Huynh et al. 2012; Corman et al. 2016; Vijgen et al. 2006; Corman et al. 2015). Coronaviruses also cause a range of infectious diseases in animal species, some with serious (economical) consequences for the livestock industry. This is illustrated by the recent emergence of a novel variant of porcine epidemic diarrhea virus, which is closely related to a strain that caused a large outbreak in China in 2010, killing almost one million piglets [for a recent review, see (Lin et al. 2016)].

The economic impact of coronavirus infections, the past and likely future emergence of pathogenic zoonotic coronaviruses and the lack of effective antiviral strategies have made it painfully clear that our preparedness to treat or prevent coronavirus infections are very limited. This highlights the importance of advancing our knowledge on the replication of these viruses and their interactions with the host.

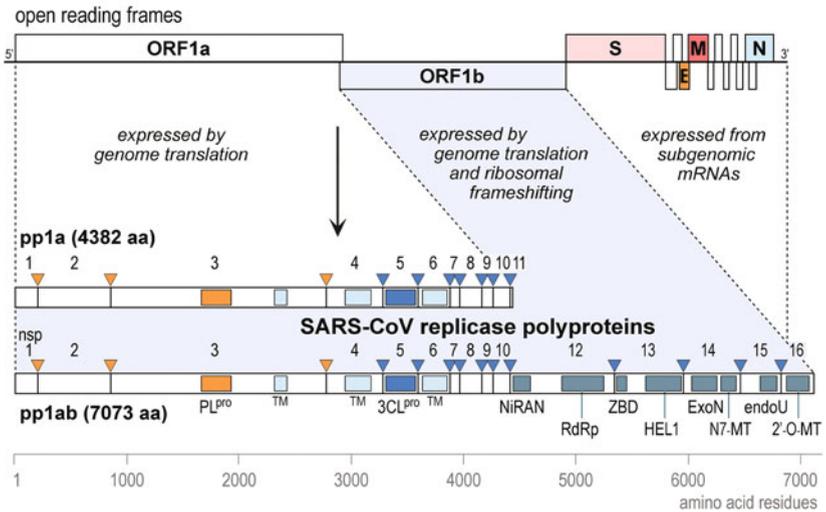
Coronaviruses are positive-stranded RNA (+RNA) viruses with, for this kind of viruses, exceptionally large genomes of ~30 kb. They have a polycistronic genome organization and employ a unique transcription mechanism to generate a nested set of subgenomic (sg) mRNAs. These are used to express the open reading frames (ORFs) located downstream of the replicase ORFs 1a and 1b (see Fig. 1a), which encode structural and accessory proteins. The sg mRNAs are 3′ co-terminal but they also contain a common 5′ leader sequence. The leader and ‘body’ segments of the sg RNAs are joined during discontinuous negative strand RNA synthesis, which produces a subgenome-length template for each of the sg mRNAs [(Sawicki and Sawicki 1995), for a recent review, see (Sola et al. 2015)].

Following entry and uncoating, the coronavirus replicative cycle (see Fig. 1a) starts with the translation of the 5′-proximal ORFs of the viral genome (ORF1a and ORF1b), which results in the synthesis of two large replicase polyproteins (pp1a and pp1ab). Synthesis of pp1ab, a C-terminally extended form of pp1a, involves a -1 ribosomal frameshift (RFS) into ORF1b occurring near the 3′ end of ORF1a. This regulatory mechanism is thought to have evolved to downregulate expression levels of ORF1b-encoded proteins compared to ORF1a-encoded nonstructural proteins (nsps) (Brierley and Dos Ramos 2006; Brierley et al. 1989). Ultimately, 15 or 16

(a)



(b)



◀**Fig. 1** Outline of the coronavirus replicative cycle and replicase polyprotein organization, based on SARS-CoV. **a** Schematic overview of the coronavirus replicative cycle. Following entry by receptor-mediated endocytosis and release of the genome into the cytosol, genome translation yields the pp1a and pp1ab replicase polyproteins. Following polyprotein cleavage by multiple internal proteases, the viral nsps assemble into an RTC that engages in minus-strand RNA synthesis. Both full-length and subgenome (sg)-length minus strands are produced, with the latter templating the synthesis of the sg mRNAs required to express the structural and accessory protein genes residing in the 3'-proximal quarter of the genome. Ultimately, novel genomes are packaged into nucleocapsids that become enveloped by budding from smooth intracellular membranes, after which the new virions leave the cell by following the exocytic pathway. See text for more details. **b** The 14 open reading frames in the genome are indicated, i.e., the replicase ORFs 1a and 1b, the four common CoV structural protein genes (S, E, M, and N) and the ORFs encoding so-called 'accessory proteins.' The bottom panel explains the organization and proteolytic processing of the pp1a and pp1ab replicase polyproteins, the latter being produced by -1 ribosomal frameshifting. The nsp3 (PL<sup>pro</sup>) and nsp5 (3CL<sup>pro</sup>) proteases and their cleavage sites are indicated in matching colors. The resulting 16 cleavage products [nonstructural proteins (nsps)] are indicated, as are the conserved replicase domains. Domain abbreviations and corresponding nsp numbers: PL<sup>pro</sup>, papain-like proteinase (nsp3); 3CL<sup>pro</sup>, 3C-like protease (nsp5); TM, transmembrane domain (nsp3, nsp4, and nsp6); NiRAN, nidovirus RdRp-associated nucleotidyl transferase (nsp12); RdRp, RNA-dependent RNA polymerase (nsp12); ZBD, zinc-binding domain (nsp13); HEL1, superfamily 1 helicase (nsp13); ExoN, exoribonuclease (nsp14); N7-MT, N7-methyl transferase (nsp14); endoU, uridylyate-specific endoribonuclease (nsp15); 2'-O-MT, 2'-O-methyl transferase (nsp16). Adopted with permission from (Snijder et al. 2016)

mature replicase proteins are released from pp1a and pp1ab due to proteolytic cleavages performed by two or three ORF1a-encoded proteases. Nsp3 contains one or two papain-like protease domains (PL1<sup>pro</sup> and PL2<sup>pro</sup>, or PL<sup>pro</sup> for SARS-CoV and infectious bronchitis virus) that process the nsp1-4 part of the replicase polyproteins. The remaining cleavage sites are processed by the viral main protease that resides in nsp5, a chymotrypsin-like enzyme also known as 3C-like protease (Snijder et al. 2016). A schematic overview of the proteolytic processing and domain structure of the SARS-CoV replicase is presented in Fig. 1b. The replicase proteins contain a variety of (enzymatic) activities and functions that are required for viral RNA synthesis and capping (Perlman and Netland 2009; Snijder et al. 2016), such as the RNA-dependent RNA polymerase (RdRp; nsp12), a helicase (nsp13), RNA cap-modifying methyltransferases (nsp14 and nsp16), and an exoribonuclease (nsp14). Together with recruited host cell proteins, the coronavirus nsps form membrane-associated replication and transcription complexes [RTCs; (van Hemert et al. 2008)], which localize to a network of virus-induced membrane structures in the perinuclear region of the infected cell (Knoops et al. 2008; Gosert et al. 2002; van der Meer et al. 1999; Brockway et al. 2003; Stertz et al. 2007; Ulasli et al. 2010). Many of the nsps appear to have multiple functions in the synthesis or processing of viral RNA, or in virus–host interactions aiming to create an optimal environment for coronavirus replication, for example by facilitating viral entry, gene expression, RNA synthesis or virus release. Moreover, to further enhance viral replication, host gene expression and antiviral defenses are targeted in several ways. Coronavirus–host interactions also play a decisive role in viral pathogenesis and the ultimate outcome of infection.

Due to the exceptional size of their +RNA genome and proteome, and the resulting complexity of the interactions with the host, our knowledge of host factors involved in coronavirus replication is still in an early stage compared to what is known for some other +RNA virus groups. In this review, we will summarize our current understanding of coronavirus–host interactions at the level of the infected cell, with special attention for the assembly and function of the viral RNA-synthesizing machinery and the evasion of cellular innate immune responses.

## 2 Host Receptors Involved in Coronavirus Entry

Entry into the target cell constitutes the first critical step in the coronavirus replication cycle. The major determinant for this step is the efficient binding of the coronavirus S glycoprotein to a protein-receptor on the cell surface. The coronavirus S protein is a type 1 glycoprotein that consists of S1 and S2 subunits and is present on the virion surface as a trimer. (Li 2016; Hulswit et al. 2016). The S1 region is involved in receptor binding and contains N- and C-terminal domains (S1-NTD and S1-CTD, respectively) (Walls et al. 2016) that may both act as receptor-binding domain (RBD), with the major determinants of cell tropism residing in S1-CTD. The elongated S2 regions form the stalk of the spike trimer and are mainly involved in triggering the fusion of the viral envelope and target cell membranes [for recent reviews on coronavirus entry and spike protein organization, see (Li 2016; Hulswit et al. 2016)].

The S1-NTD is mainly involved in facilitating virus binding and entry, by interacting with glycans on the host cell surface. Based on the crystal structure of the betacoronavirus S1-NTD and the sequence conservation among the S1-NTDs of other coronaviruses, all coronavirus S1-NTDs are thought to share a galectin fold that mediates binding to sialic acids, like N-glycolylneuraminic acid (Neu5Gc), N-acetylneuraminic acid (Neu5Ac), and/or 5-N-acetyl-9-O-acetylneuraminic acid (Neu5,9Ac2) (see (Li 2016), and references herein). An exception is the murine hepatitis virus (MHV) S1-NTD, which binds the N-terminal D1 domain of carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), a type-I membrane protein belonging to the immunoglobulin superfamily (Walls et al. 2016; Williams et al. 1991).

To mediate entry into host cells, the S1-CTD of most known members of the alphacoronavirus genus interacts with aminopeptidase N (APN) (for an overview and references, see Table 1). However, the alphacoronavirus HCoV-NL63 uses a different type-I membrane glycoprotein, angiotensin-converting enzyme 2 (ACE2) (Wu et al. 2009), which contains a large N-terminal ectodomain composed of two alpha-helical lobes. The same molecule, ACE2, has been identified as a receptor for the zoonotic betacoronavirus SARS-CoV (Li et al. 2003). The betacoronaviruses MERS-CoV and bat coronavirus HKU4 use yet another cellular peptidase for virus entry: dipeptidyl peptidase 4 (DPP4) (Yang et al. 2014; Raj et al. 2013). The MERS-CoV S protein has a higher affinity for human DPP4, while the HKU4 S

**Table 1** Overview of known coronavirus entry receptors

Genus	Species:	S1-NTD	S1-CTD	References
Alphacoronavirus	Alphacoronavirus 1	Neu5Gc and Neu5Ac*	APN	(Tresnan et al. 1996; Delmas et al. 1992)
	PEDV	Neu5Ac	APN	(Liu et al. 2015; Li et al. 2007)
	PRCV		APN	(Schultze et al. 1996)
	HCoV-229E		APN	(Yeager et al. 1992)
	HCoV-NL63		ACE2	(Wu et al. 2009)
Betacoronavirus	Betacoronavirus 1	Neu5,9Ac2		(Schultze and Herrler 1992; Krempf et al. 1995)
	MERS-CoV		DPP4	(Raj et al. 2013)
	MHV	CEACAM1		(Williams et al. 1991)
	HKU1	Neu5,9Ac2		(Huang et al. 2015b)
	HKU4		DPP4	(Yang et al. 2014)
	SARS-CoV		ACE2	(Li et al. 2003)
Gammacoronavirus	IBV	Neu5Gc		(Schultze et al. 1993)
Deltacoronavirus	PDCV	Unknown	unknown	

(Abbreviations PEDV Porcine epidemic diarrhea virus; TGEV Transmissible gastroenteritis coronavirus; PRCV Porcine Respiratory coronavirus; FCoV Feline coronavirus; CCoV Canine coronavirus; HCoV Human coronavirus; BCoV Bovine coronavirus; MHV Murine hepatitis virus; IBV Infectious bronchitis virus; PDCV Porcine delta coronavirus). \*Within the alphacoronavirus 1 species, only for TGEV the sialic acids Neu5Gc and Neu5Ac has been identified as attachment factors

protein binds more strongly to bat DPP4 (Yang et al. 2014). Chemical peptidase inhibitors do not affect virus entry, indicating that SARS-CoV and MERS-CoV receptor usage and entry are independent of the receptor's peptidase activity and merely depend on binding to these particular host receptors (Li et al. 2005c; Raj et al. 2013).

Besides the receptors discussed above, also extracellular, cell surface-associated and/or lysosomal proteases play a role in coronavirus entry by activating the fusion activity of the S protein [for a recent review, see (Li 2016)]. For SARS-CoV, fusion of the viral and cellular membrane is triggered upon cleavage of the S protein by the cell surface-associated transmembrane protease, serine 2 (TMPRSS2) (Glowacka et al. 2011). The same protease is important for cleavage and activation of the HCoV-229E and MERS-CoV S protein (Shirato et al. 2013; Bertram et al. 2013). After endocytosis, the SARS-CoV S protein is cleaved by the lysosomal proteases cathepsin L and cathepsin P in early endosomes, leading to fusion of the virus envelop with the endosome membranes and release of the viral RNA into the cytosol of the infected cell (Huang et al. 2006a, b; Simmons et al. 2005). MERS-CoV entry occurs by a similar mechanism (Shirato et al. 2013; Burkard et al. 2014), although inhibition of the cellular protease furin abolished the entry of MERS-CoV but not SARS-CoV, indicating that furin-mediated cleavage is pivotal

for efficient MERS-CoV entry (Burkard et al. 2014; Follis et al. 2006). On the other hand, MHV strain A59 was shown to fuse with late endosomes and to depend on their low pH for S protein cleavage (Burkard et al. 2014). Therefore, it has been proposed that coronavirus fusion with endosomes depends on the use of a furin cleavage site just upstream of the fusion peptide (Burkard et al. 2014). However, why some coronaviruses fuse with early endosomes and others with late endosomes, and whether these events play a role in host tropism and pathogenicity, is still not completely understood (Burkard et al. 2014). The complexity of S protein cleavage is further highlighted by a recent paper by Park et al., which clearly showed that MERS-CoV entry depends on furin-mediated cleavage in virus-producing cells. Subsequently, cleaved MERS-CoV S proteins could be processed by proteases on recipient cells and virions could enter the cells via early endosomes or even by fusing with the plasma membrane. MERS-CoV virions that contain uncleaved S proteins may rather fuse with late endosomes (Park et al. 2016).

The interaction of the coronavirus S glycoprotein with its cell surface receptor is a key determinant for host tropism. In the case of SARS-CoV, only a few mutations (N479L and T487S) in the S protein's RBD sufficed to dramatically increase the affinity for human ACE2 (Li 2008). Likewise, the MERS-CoV S protein contains two mutations compared to the bat coronavirus HKU4 S protein, which can bind the human DPP4 receptor, but cannot mediate viral entry due to lack of activation by human proteases. The two mutations in the MERS-CoV S protein (S746R and N762A) enable cleavage by the human proteases and thus viral entry into human cells and may have contributed to the zoonotic transfer of MERS-CoV (Yang et al. 2015).

Several lineage A betacoronaviruses also carry a hemagglutinin-esterase (HE) protein on their surface. HE proteins contain a lectin-binding domain that mediates binding to O-acetylated sialic acids, while also possessing sialate-O-acetylsterase receptor-destroying enzyme activity targeting these same glycans on the cell surface. The receptor-destroying enzyme activity is thought to prevent attachment to non-permissive cells, while the HE protein also facilitates entry into target cells after binding to the main entry receptor (Zeng et al. 2008; Langereis et al. 2010; Bakkers et al. 2016).

### **3 Translation and the Unfolded Protein Response in Coronavirus-Infected Cells**

All viruses depend on the host cell's translation machinery for the production of their proteins and infectious progeny. Moreover, protein synthesis is also pivotal for the host cell's response to infection by mounting an antiviral (innate) immune response. Hence, it is not surprising that many +RNA viruses modulate host protein synthesis in order to limit the translation of cellular mRNAs and favor the synthesis of viral proteins [reviewed in (Walsh and Mohr 2011; Fung et al. 2016)].

In eukaryotic cells, translation is initiated by formation of the heterotrimeric eIF2 complex, which is composed of the regulatory  $\alpha$ -subunit, the tRNA-binding  $\beta$ -subunit, and a GTP-binding  $\gamma$ -subunit. The eIF2 complex is responsible for loading of the 40S subunit with Met-tRNA<sub>i</sub>. After mRNA binding, this 43S complex serves as a scaffold for the recruitment of several additional proteins, including eIF3, to the capped 5' end of the mRNA. Subsequently, the cap-binding eukaryotic translation initiation factor 4F (eIF4F) joins this pre-initiation complex (48S complex), which then scans the mRNA in the 5' to 3' direction to localize a translation initiation codon. At this point, the 60S ribosomal subunit joins and protein synthesis starts [reviewed in (Jackson et al. 2010)]. Polyadenine-binding protein (PABP), which binds to the poly(A)-tail of mRNAs, is also involved in stimulating protein synthesis.

The eIF2 complex can be inactivated by phosphorylation of its alpha subunit (eIF2 $\alpha$ ) by one of four mammalian kinases in response to various (external) triggers. These kinases are eIF2 $\alpha$  kinase 4 (also known as GCN2), heme-regulated inhibitor (HRI), PKR-like endoplasmic reticulum kinase (PERK), which is activated upon induction of ER stress, and double-stranded (ds) RNA-activated protein kinase (PKR).

Since several stages of the coronavirus replication cycle are closely associated with the endoplasmic reticulum (ER), ER stress is thought to occur during coronavirus infection. Indeed, expression of several coronavirus proteins, including the heavily glycosylated S protein, was shown to induce ER stress, which was also observed in coronavirus-infected cells [(Chan et al. 2006), and reviewed in (Fung et al. 2016)]. Consequently, the unfolded protein response (UPR) is induced, which alleviates the problems by inhibiting translation (by PERK-induced phosphorylation of eIF2 $\alpha$ ), stimulating protein folding, and eventually triggering apoptosis. Compared to, for example, hepatitis C virus [see review by (Chan 2014)], many details of how coronaviruses control the UPR remain unknown, but they generally seem to manipulate PERK activity to control the level of translation [reviewed by (Fung et al. 2016)].

PKR is a serine/threonine protein kinase that is activated by the presence of dsRNA, a hallmark of RNA virus infection. PKR is a key player in the innate immune response to RNA virus infection as it upregulates antiviral gene expression, including the production of interferons (IFNs). Coronaviruses have evolved various strategies to counteract PKR-mediated signaling in order to prevent the translational shut-off due to eIF2 $\alpha$  phosphorylation. For example, infectious bronchitis virus (IBV) appears to (weakly) antagonize PKR by blocking its activation as well as inducing the expression of growth arrest and DNA-damage-inducible 34 protein (GADD34), leading to reduced eIF2 $\alpha$  phosphorylation in IBV-infected cells (Wang et al. 2009). Upon MHV infection, sustained eIF2 $\alpha$  phosphorylation and repression of GADD34 expression leads to translational repression of cellular mRNAs, which may be beneficial for MHV infection (Bechill et al. 2008). Recently, the MERS-CoV ORF4a protein was shown to counteract the PKR-induced formation of stress granules, probably by binding viral dsRNA to shield it from detection by PKR, thereby preventing translational inhibition (Rabouw et al. 2016). Also

transmissible gastroenteritis virus (TGEV) has been reported to modulate host cell translation, in this case through its protein 7, which promotes eIF2 $\alpha$  dephosphorylation through an interaction with protein phosphatase 1 (PP1), a key regulator of the host's antiviral response (Cruz et al. 2011). The S proteins of both SARS-CoV and IBV were found to physically interact with eIF3F, to modulate host translation, including the expression of the pro-inflammatory cytokines interleukin (IL) 6 and 8, at a later stage of infection (Xiao et al. 2008). Therefore, this interaction may play an important regulatory role in coronavirus pathogenesis.

Besides modulating eIF2 $\alpha$  phosphorylation, coronaviruses have other ways of manipulating the translation machinery. Importantly, the nsp1 proteins of both alpha- and betacoronaviruses were identified as inhibitors of multiple steps of translation initiation (Lokugamage et al. 2012, 2015). SARS-CoV nsp1 does so by inhibiting 48S initiation complex formation and interfering with its conversion into the 80S initiation complex (Lokugamage et al. 2012). In addition, the multifunctional SARS-CoV nsp1 is able to directly bind the 40S ribosomal subunit to inhibit its function in translation (Kamitani et al. 2009). Moreover, this complex of nsp1 and the 40S subunit induces cleavage of cellular mRNAs to suppress host cell translation to an even larger extent (Kamitani et al. 2006). MERS-CoV nsp1 seems to act differently, by selectively inhibiting the translation of mRNAs produced in the nucleus, while leaving the translation of the cytosolically made viral mRNAs unaffected (Lokugamage et al. 2015). The difference with SARS-CoV nsp1 is further highlighted by the observation that MERS-CoV nsp1 does not bind to the 40S ribosomal subunit (Lokugamage et al. 2015).

Taken together, several coronavirus studies have highlighted how modulation of host protein synthesis through different—often parallel—mechanisms can have a profound effect on the cell. In this manner, viral ‘translation modulators’ may contribute importantly to coronavirus pathogenicity.

## 4 Coronavirus-Induced Modification of Host Cell Membranes

As outlined in Chap. 1, a common characteristic of +RNA viruses is that their RNA synthesis takes place in the cytoplasm and is associated with virus-induced structures derived from cellular endomembranes [reviewed in (Romero-Brey and Bartenschlager 2016; Reid et al. 2015; van der Hoeven et al. 2016)]. This is an intriguing kind of virus–host interaction and the architecture of these ‘replication organelles’ has now been studied in detail for quite a number of viruses. Nevertheless, their exact functions have remained largely obscure. In general, two types of +RNA virus-induced membrane structures have been recognized [recently reviewed by (van der Hoeven et al. 2016)]. The first type is characterized by single-membrane spherules, invaginations with a negative curvature formed in the membranes of organelles such as the endoplasmic reticulum (ER), peroxisomes, or

endosomes, with the source of the membrane depending on the virus under study. The viral replication machinery is located within these spherules and RNA products are exported through a channel that connects the spherule's interior and the cytosol, so that they can engage in translation or particle assembly. Flaviviruses and alphaviruses are examples of virus families inducing the formation of this type of replication organelles [reviewed in (den Boon and Ahlquist 2010)].

The second type of replication structures, which includes those found in coronavirus-infected cells, is dominated by double-membrane vesicles (DMVs), often accompanied by other structures such as tubules, zippered ER and/or convoluted membranes, together forming a reticulovesicular network in the cytosol (Knoops et al. 2008; Maier et al. 2013; Ulasli et al. 2010; Hagemeyer et al. 2012; Gosert et al. 2002) (Fig. 1c). Picornaviruses, arteriviruses, and flaviviruses like hepatitis C virus (HCV) induce similar structures [reviewed in (van der Schaar et al. 2016; van der Hoeven et al. 2016; Paul et al. 2014), respectively]. It is generally thought that viral nsps that have transmembrane regions, or are otherwise anchored to membranes, drive the formation of these structures. In the case of coronaviruses, nsp3, nsp4, and nsp6 have been implicated in this process (Angelini et al. 2013; Hagemeyer et al. 2012).

In terms of host factors and pathways involved in the formation of coronavirus replication organelles, a variety of hypotheses and data sets have been put forward. Much of this data is still under debate though, making it quite difficult to formulate a consensus theory. The involvement of ER membranes seems to be generally accepted and is supported by the presence of ribosomes and ER markers such as sec61 $\alpha$  and protein disulphide isomerase (PDI) on the surface of or inside virus-induced double-membrane structures, and the fact that the outer membrane of coronavirus-induced DMVs can be continuous with ER cisternae (Hagemeyer et al. 2014; Knoops et al. 2008, 2010; Snijder et al. 2006). In accordance with this link to the ER, studies by de Haan and co-workers (Oostra et al. 2007; Verheije et al. 2008) suggested that the secretory pathway, including coatomer protein (COP)-dependent processes and associated factors such as GBF-1, plays an important role in replication. However, no co-localization was observed between nsp4, a marker for the coronavirus-induced membrane structures, and secretory pathway markers. These observations are in agreement with the results of our own siRNA screen for host factors influencing SARS-CoV replication, which identified COPB2 (or  $\beta'$ -COP), a subunit of the coatomer protein complex, as a strong proviral (or 'dependency') factor (de Wilde et al. 2015). Depletion of COPB1 and GBF1, which are part of this same machinery, also severely affected SARS-CoV replication, confirming that the integrity of the secretory pathway is important for replication (de Wilde et al. 2015; Knoops et al. 2010). Several reports have described the importance of phosphatidylinositol 4-kinases (PI4Ks) in +RNA virus replication, which was first discovered through siRNA screens searching for cellular factors important for picornavirus and hepatitis C virus replication (Reiss et al. 2011; Hsu et al. 2010; Berger et al. 2009). These kinases seem to be recruited to the sites of membrane modification and stimulate the production of PI4P lipids, which together supports the formation and/or functionality of viral replication structures. The underlying

mechanism is not exactly clear yet, and several hypotheses have been put forward [reviewed in (Altan-Bonnet and Balla 2012)]. Also for SARS-CoV replication one of the PI4K isoforms, PI4KIIIbeta, was shown to be important (Yang et al. 2012), although it seems to play a role in entry rather than later steps of the replication cycle. However, in our kinome-based siRNA screen, PI4Ks were not identified as cellular factors involved in SARS-CoV replication (de Wilde et al. 2015), although siRNA screens are known to yield false-negative results.

A long-standing hypothesis regarding coronavirus-induced double-membrane structures is the possible involvement of the autophagy pathway, which derives from the fact that autophagosomes also have double membranes. Some reports suggested that coronaviruses hijack the autophagy machinery for DMV biogenesis in support of their replication (de Haan and Reggiori 2008; Prentice et al. 2004; Maier and Britton 2012). However, another study showed that the essential autophagy factor Atg5 is not required at all for coronavirus replication in primary cells (Zhao et al. 2007). Molinari and co-workers then proposed that so-called EDEMosomes are being hijacked for the formation of coronavirus membrane structures (Reggiori et al. 2010). These EDEMosomes are defined as single-membrane vesicles that pinch off from the ER to remove ERAD regulators (like EDEM1 and OS-9) when this is needed to tune the ERAD machinery (Cali et al. 2008). The process seems a deviation from the autophagy pathway, with the EDEMosomes accumulating LC3-I, a form that is inactive in the canonical autophagy pathway. Reggiori and co-workers claimed that coronaviruses hijack these vesicles to form their reticulovesicular network, and this hypothesis was later extended to arteriviruses (Monastyrska et al. 2013). However, several questions have remained unanswered and other published data appear to be at odds with the EDEMosome hypothesis. For example, it has remained entirely unclear how the small single-membrane EDEMosome vesicles would be converted into the elaborate network of (much larger) DMVs, convoluted membranes (CM), and other structures that are typical of coronavirus-infected cells. Furthermore, EDEMosomes have been characterized as alternative transport vesicles that explicitly are not associated with COP-coats and are independent of the canonical secretory pathway, which—as Reggiori and co-workers argued—may explain why secretory pathway markers do not localize to replication membranes (Reggiori et al. 2010). Nonetheless, the integrity of the secretory pathway and the function of COP components clearly influences coronavirus replication (Verheije et al. 2008; Oostra et al. 2007; Knoops et al. 2010; de Wilde et al. 2015).

All in all, although a variety of studies addressed the possible host pathways and factors involved in the formation and function of the replication-associated membrane structures, our understanding of coronavirus replication organelle biogenesis is still far from complete. Possibly, the expansion of our basic knowledge regarding relevant cellular processes, such as membrane trafficking and autophagy, may provide more clues on the molecular mechanisms underlying these interesting interactions of coronaviruses with their host cells.

## 5 Host Proteins Interacting with the Coronavirus Genome and Its Replication or Expression

The 5'- and 3'-proximal regions of coronavirus RNAs contain key regulatory elements for their RNA synthesis [for a recent review, see (Yang and Leibowitz 2015)]. Although in general the precise role of host factors interacting with these signals is poorly understood, RNA-binding proteins have been identified as frequently used enhancers of coronaviral RNA synthesis (Table 2). Both termini of the coronavirus genome fold into higher-order RNA structures, which presumably stabilize the molecule and are also involved in inter- and intramolecular interactions that facilitate viral replication (Brian and Baric 2005). Viral and cellular proteins can bind to these structures to drive or modulate translation, replication, and subgenomic RNA synthesis.

The cellular protein polypyrimidine tract-binding protein (PTB; or heterogeneous ribonucleoprotein protein (hnRNP) I) was found to bind the 5' leader sequence of the TGEV (Galan et al. 2009) and MHV genome (Li et al. 1999; Choi et al. 2002). In the case of MHV, PTB was found to bind a 5'-proximal pentanucleotide UCUAA repeat and to be critical for RNA synthesis. HnRNP Q, or SYNCRIP, also binds the 5'-proximal part of the MHV genome and its knockdown reduced MHV RNA synthesis and virus replication. The case for a specific role in RNA synthesis was strengthened by the observation that neither overexpression nor downregulation affected translation of MHV RNA (Choi et al. 2004). Zinc finger CCHC-type and RNA-binding motif 1 (MADP1) was shown to bind the 5' end of the SARS-CoV and IBV genome (Tan et al. 2012). In analogy to SYNCRIP, silencing of MAPD1 reduced IBV replication by interfering with viral RNA synthesis, showing that MAPD1 plays a proviral role in the coronavirus cycle (Tan et al. 2012).

In addition to proteins that bind the 5' UTR of the coronavirus RNA, many RNA-binding proteins have been identified that interact with the 3' UTR and/or poly(A)-tail, although the role of these proteins is poorly understood. First, mitochondrial aconitase, stabilized by a complex of heat-shock protein (hsp) 40, hsp60, and mitochondrial hsp70, binds the 3'-terminal 42 nucleotides of the MHV 3' UTR, just upstream the poly(A) tail (Nanda et al. 2004; Nanda and Leibowitz 2001). The protein p100 coactivator was found to bind the TGEV 3' UTR and/or poly(A) tail (Galan et al. 2009) and the PABPs interact with the TGEV, BCoV and IBV genome to promote its efficient replication (Spagnolo and Hogue 2000; Galan et al. 2009; Emmott et al. 2013). It has been proposed that binding of PABPs to the viral RNA ensures efficient translation and mRNA stability (Enjuanes 2005). In addition, by using an RNA-affinity purification and mass spectrometry approach, Galan et al. (2009) identified several hnRNPs that bind the 3'-terminal ~500 nucleotides of the TGEV genome. Several of these are presumed RNA-binding proteins for other coronaviruses and were proposed to play a role in viral RNA synthesis (Table 2). However, the exact function and relevance of hnRNPs and other RNA-binding proteins in coronavirus replication is still not fully understood. The majority of RNA-binding proteins were identified in co-immunoprecipitation studies that made

**Table 2** Overview of RNA-binding proteins that have been described to interact with the RNA of various coronaviruses

Protein	Coronavirus	Cellular function	Interactions or proposed function in viral RNA synthesis	Experimental evidence	References
Annexin A2	IBV	Cellular RNA-binding protein	Modulates IBV frameshifting efficiency	In vitro pull-down	(Kwak et al. 2011)
hnRNP A0	TGEV	Involved in RNA splicing	Binds the TGEV 3' UTR or poly(A)-tail	In vitro pull-down	(Galan et al. 2009)
hnRNP A1	MHV	Involved in RNA transport, processing, and splicing	High affinity for the MHV (-)-strand leader RNA of the MHV genome. Also binds the intergenic region that regulates ORF7 synthesis Binds the MHV 3'-UTR, may mediate RNP formation to bind 5'end and 3'end of the MHV genome together with PTB. Binds the TGEV 3' UTR or poly(A)-tail	In vitro pull-down In vitro pull-down	(Li et al. 1997; Zhang and Lai 1995; Furuya and Lai 1993) (Huang and Lai 1999, 2001) (Galan et al. 2009)
hnRNP A2-B1	TGEV, MHV	Involved in RNA transport and splicing	hnRNP A1 regulates MHV RNA synthesis Binds the TGEV 3' UTR or poly(A)-tail and the (-)-strand MHV leader RNA	Overexpression In vitro pull-down	(Shi et al. 2000) (Galan et al. 2009; Shi et al. 2003)
hnRNP A-B and A3	MHV	Involved in RNA transport and splicing	Role in MHV RNA synthesis	In vitro pull-down	(Shi et al. 2003)
hnRNP Q (SYNCRIP)	TGEV, MHV	Involved in RNA processing and splicing	Binds the TGEV 3' UTR or poly(A)-tail and the MHV 5'end.—Involved in MHV RNA synthesis	In vitro pull-down, virus infection	(Galan et al. 2009; Choi et al. 2004)
hnRNP U	TGEV	Involved in RNA processing and splicing	Binds the TGEV 3' UTR or poly(A)-tail	In vitro pull-down	(Galan et al. 2009)

(continued)

Table 2 (continued)

Protein	Coronavirus	Cellular function	Interactions or proposed function in viral RNA synthesis	Experimental evidence	References
MADP1	SARS-CoV, IBV	Involved in RNA splicing	Binds the 5' UTR of the viral genome. Functions in IBV RNA synthesis	In vitro pull-down, virus infection	(Tan et al. 2012)
Mitochondrial aconitase, hsp40, hsp60, mtHsp70	MHV	Mitochondrial aconitase is a component of the citric acid cycle, Hsp40: chaperone, regulate function of hsp70. Mt-hsp70: chaperone, helps to protect from cell stress. Hsp60: mitochondrial chaperone	Hsp40, hsp60, mtHsp70 stabilize complex with mitochondrial aconitase, binds the last 42 nucleotides of the 3' UTR of MHV	In vitro pull-down, virus infection	(Yu and Leibowitz 1995; Nanda and Leibowitz 2001; Nanda et al. 2004)
NONO	IBV	RNA-binding protein which plays a role transcriptional regulation and RNA splicing	Interacts indirectly with the IBV nucleocapsid protein via viral and/or cellular RNA	In vitro pull-down, SILAC	(Emmott et al. 2013)
p100 kDa coactivator	TGEV	Involved in transcription and RNA interference	Binds the TGEV 3' UTR or poly(A)-tail	In vitro pull-down	(Galan et al. 2009)
PABPs	BCoV, TGEV, IBV	RNA-binding protein that binds to the poly(A) tail of cellular mRNA. Involved in mRNA translation	Binds to the poly(A)-tail of the viral genome. Signal for genome replication	In vitro pull-down, SILAC	(Spagnolo and Hogue 2000; Galan et al. 2009; Emmott et al. 2013)
PTB (hnRNP I)	TGEV, MHV	Involved in RNA splicing	Binds the 5' end leader sequence of the viral genome. (MHV: binds 5' pentanucleotide repeat UCUAA). May form RNP complex with hnRNP A1, MHV N and viral RNA. Regulates viral transcription	In vitro pull-down, overexpression	(Galan et al. 2009; Li et al. 1999; Choi et al. 2002; Huang and Lai 1999)

(Abbreviations PABP poly(A)-binding protein; PCBP poly-r(C)-binding protein; FCBP poly-r(C)-binding protein; MADP1: zinc finger CCHC-type and RNA-binding motif 1; DDX DEAD-box protein; BCoV bovine coronavirus; SYNCRIP synaptotagmin-binding cytoplasmic RNA-interacting protein; NONO Non-POU domain-containing octamer-binding protein; SILAC stable isotope labeling with amino acids in cell culture)

use of *in vitro* transcribed RNA and the importance of many of these factors has not been tested in the context of virus replication. For example, hnRNP A1 was reported to have affinity for the complement of the MHV leader sequence and the TRS that regulates mRNA<sup>7</sup> synthesis (Li et al. 1997; Zhang and Lai 1995). Interestingly, however, Shen and Masters (Shen and Masters 2001) showed that MHV replicates equally well in hnRNP A1<sup>-/-</sup> CB3 cells and parental CB3 cells, which lack and express hnRNP A1, respectively. This strongly suggests that hnRNP A1 itself is not pivotal for MHV replication. On the other hand, Lai and co-workers showed that multiple hnRNPs can bind to the same viral RNA and postulated that hnRNPs may be able to functionally substitute each other in MHV infection (Shi et al. 2003). These apparent contradictions also highlight the technical complexity of dissecting the precise role of RNA-binding proteins in coronavirus replication.

On a different level, RNA-binding proteins were investigated as potential modulators of the efficiency of the coronavirus ORF1a/1b frameshift event during replicase gene translation. For example, annexin A2 binds the IBV slippery sequence, on which ribosomes are repositioned and engage in expression of ORF1b. Host proteins may also affect the activity of the viral RTC indirectly. Using an *in vitro* assay, the RNA-synthesizing activity of semi-purified SARS-CoV RTCs was shown to depend on an as yet unidentified cytosolic host factor, which is not directly associated with the viral RTC (van Hemert et al. 2008).

## 6 Host Innate Immune Responses Against Coronaviruses, and Viral Countermeasures

Cells generally respond to a virus infection by mounting an innate antiviral response to limit the spread of the infection and aid in inducing an adaptive immune response that will eventually clear the virus. Many viruses have evolved strategies to suppress and/or evade these (innate) immune responses, which can dramatically influence the course of the infection, including pathogenesis and persistence in the host. In the case of +RNA viruses, the innate immune system is often triggered by the dsRNA and 5'-triphosphate-bearing RNA molecules that arise as replication intermediates in the cytosol. These molecules are foreign to the cell and can be recognized by the intracellular sensors of the Rig-I-like receptor (RLR) family, such as retinoic acid-inducible gene 1 (RIG-I) and melanoma differentiation-associated protein 5 (MDA-5) which are expressed in almost all cells [reviewed in (Wilkins and Gale 2010; Bruns and Horvath 2014)]. For recognition of coronavirus RNAs, MDA-5 seems the most important cytosolic sensor (Zust et al. 2011; Zalinger et al. 2015; Kindler and Thiel 2014), although in some cell types RIG-I also seems to play a role (Li et al. 2010). Also the toll-like receptors which are expressed on the cell surface or reside in the endosomes of immune cells can recognize viral nucleic acids or proteins. TLR3 plays a role in the recognition of coronaviruses (Totura et al. 2015; Mazaleuskaya et al. 2012) and also TLR4 was shown to be relevant

during MHV infection in mice (Khanolkar et al. 2009). Recently it was shown that the SARS-CoV M protein is recognized via a TLR-like pathway that is independent of the canonical TRAF3-mediated signaling pathway (Wang and Liu 2016). Activation of one or more of these sensors generally leads to the activation of the transcription factors IFN-regulatory factor 3 and 7 (IRF3, IRF7) and NF- $\kappa$ B. These stimulate the expression and excretion of Type-I IFN and pro-inflammatory cytokines, which in turn activate the JAK-STAT signaling cascade that induces the expression of a myriad of antiviral interferon-stimulated genes (ISGs). This ultimately results in an antiviral state of the infected cell, as well as neighboring cells. ISGs were shown to target virtually all steps of the viral cycle in order to restrict viral replication (Schoggins and Rice 2011). The p38 mitogen-activated protein kinases (MAPKs) play a role in the induction of inflammatory cytokines IL-6 and IL-8, and were linked to countering coronavirus infections through several studies [reviewed in (Mizutani 2007)]. 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, although it has remained unclear which viral protein(s) is responsible (Liao et al. 2011). Innate immune and inflammatory signaling pathways are extensively regulated in order to prevent adverse effects of their over-stimulation. Apart from phosphorylation and other regulatory mechanisms, the system is controlled by ubiquitination at numerous points in the signal transduction cascade. For example, RIG-I, TANK-binding kinase 1 (TBK1), and TNF receptor-associated factor 3 (TRAF3) were shown to be activated by Lys63-linked ubiquitination (Jiang and Chen 2012).

The importance of the innate immune system in the context of coronavirus infections can be illustrated in at least three ways. First, in severe cases of SARS the pathology was associated with aberrant or hyper-activation of innate immune signaling. This resulted in the aberrant production of interferons and high levels of pro-inflammatory cytokines such as IL-1, IL-6, IL-8, CXCL-10 and TNF-alpha in the lungs [reviewed in (Totura and Baric 2012)]. Interestingly, a systems biology study that evaluated the transcriptome after infection of cultured cells with two different human MERS-CoV isolates (MERS-CoV Eng 1 vs. SA 1) suggested that viral sequence differences relate to variations in innate immune evasion, which may in turn result in different immune responses. These differences may link to differential STAT3 activation leading to activation or inhibition of, *e.g.*, IFN, NF- $\kappa$ B, and IRF7 (Selinger et al. 2014). Second, as described in Chap. 4, coronaviruses employ elaborate mechanisms to shield the viral replication machinery from the innate immune sensors in the cytosol. Third, besides the presumed shielding of viral PAMPs by the replication organelles, coronaviruses seem to encode numerous gene products that actively counteract or help to circumvent innate immune responses [reviewed in (Totura and Baric 2012; Kindler and Thiel 2014; Vijay and Perlman 2016)].

To illustrate the multitude of activities coronaviruses employ to actively suppress innate immunity, and the diversity of viral gene products involved, some examples are discussed below. First, besides inhibiting cellular mRNA translation (see paragraph above), SARS-CoV nsp1 was shown to block IFN signaling by

reducing the amount of phosphorylated STAT1 (p-STAT1) in infected cells (Wathelet et al. 2007). Also nsp1 proteins of other alpha- and betacoronaviruses were shown to inhibit type-I IFN signaling, mostly through the host-shut-off activity of this N-terminal subunit of the coronavirus replicase polyprotein (reviewed in Narayanan et al. 2015). Further downstream in the pp1a polyprotein, the papain-like protease 2 domain (PL2<sup>pro</sup>) of many coronaviruses and the TGEV PL1<sup>pro</sup> domain, which reside in nsp3, exhibit deubiquitination (DUB) activity in biochemical experiments [reviewed in (Mielech et al. 2014)]. This DUB activity may remove ubiquitin from innate immune signaling factors to suppress the induction of an antiviral state, and indeed was shown to reduce IFN signaling in biochemical experiments using PL2<sup>pro</sup> overexpression for several coronaviruses, including SARS-CoV (Matthews et al. 2014a; Li et al. 2016) and MERS-CoV (Chen et al. 2007; Clementz et al. 2010; Bailey-Elkin et al. 2014). Similarly, experiments suggested that MHV PL2<sup>pro</sup> deubiquitinates and binds TBK1, as well as IRF3 (Zheng et al. 2008; Wang et al. 2011). For the distantly related arterivirus EAV, infection with a mutant lacking a similar papain-like protease-driven DUB activity resulted in an increased innate immune response after infection, indicating that the viral DUB activity indeed has a function in suppression of innate immune response during infection (van Kasteren et al. 2013). Adjacent to the PL2<sup>pro</sup> domain, some coronaviruses contain a domain originally coined ‘SARS-CoV unique domain’ (SUD). The two domains together were recently also implicated in innate immune suppression by binding and stimulating a cellular ubiquitin E3 ligase, RCHY1, resulting in augmented degradation of p53 (Ma-Lauer et al. 2016). The main protease, nsp5, of PEDV was shown to cleave NEMO, an important innate immune regulator protein (Wang et al. 2015). When independently expressed, coronavirus nsp6, which is probably one of the most hydrophobic proteins encoded in the genome, seems to induce and/or influence autophagy. However the relevance of this observation for virus-infected cells and possible links to innate immune responses against coronaviruses remain to be investigated (Cottam et al. 2014; Cottam et al. 2011). A conserved domain in the coronavirus nsp16 directs 2'-O methylation of the viral RNA, thereby preventing its recognition by MDA5 (Menachery et al. 2014; Züst et al. 2011).

Besides conserved replicase subunits, also several of the less-conserved products of downstream open reading frames, i.e., the so-called ‘accessory proteins’, have innate immunity suppressing features. In fact, these proteins may have been acquired by different coronavirus lineages for this purpose, since they are generally dispensable for replication in cell culture. The phosphodiesterase (ns2) encoded by ORF2a in MHV and related viruses antagonizes RNase L activation, and this was shown to be important for replication in natural host cells (Zhao et al. 2012; Li and Weiss 2016). Observations supporting a role in blocking IFN production and/or signaling were also made for the ORF3b and ORF6 proteins of SARS-CoV (Kopecky-Bromberg et al. 2007) and the ORF3b proteins of other SARS-like coronaviruses (Zhou et al. 2012). Also the proteins encoded by MERS-CoV ORFs 4a, 4b and 5 (Siu et al. 2014; Yang et al. 2013; Niemeyer et al. 2013; Matthews et al. 2014b) all seem to block IFN signaling, although many of the initial reports

were based only on overexpression experiments, which should be interpreted with caution. Recently however, more insight into the mechanisms by which some of these viral proteins suppress antiviral responses was obtained. Like MHV ns2 (see above), the MERS-CoV ORF4b protein and its homologs from other betacoronaviruses were shown to have phosphodiesterase activity. They can block activation of the RNase L-mediated innate immune response during infection by degrading 2',5'-oligoadenylate, which is the activator of RNase L (Thornbrough et al. 2016). Additionally, MERS-CoV ORF4a prevents activation of the PKR-stress granule route by binding dsRNA, in the context of an infection (Rabouw et al. 2016). The SARS-CoV ORF6 protein was shown to block p-STAT1 import into the nucleus by interacting with the nuclear pore, and this block was suggested to reduce innate immune responses and the expression of genes that affect virus-induced pathogenesis (Frieman et al. 2007; Kopecky-Bromberg et al. 2007; Huang et al. 2015a). Interestingly, MERS-CoV, along with the other coronaviruses not belonging to the SARS-CoV cluster, lacks an ORF6 homolog, and since MERS-CoV is also more sensitive to treatment with type-I interferons, it was hypothesized that this could—in part—be explained by this difference (de Wilde et al. 2013b). Also the coronaviral structural proteins seem to possess innate immunity-modulating activities, although also here most studies involved overexpression experiments. The SARS-CoV and MERS-CoV M proteins bind TRAF3 to prevent its binding to TBK1 and eventually to prevent nuclear translocation of this complex, which blocks IRF3-mediated signaling (Siu et al. 2009; Lui et al. 2016). Overexpression experiments showed a negative impact of the SARS-CoV N protein on (the early stages of) innate immune signaling (Frieman et al. 2009; Kopecky-Bromberg et al. 2007; Lu et al. 2011). MHV N protein also seems to counteract type-I IFN signaling, and could do this in the context of a recombinant vaccinia virus infection (Ye et al. 2007). Purified SARS-CoV spike (S) protein stimulates inflammatory and other innate responses, possibly through TLR2 activation (Dosch et al. 2009). Finally, the SARS-CoV E protein, is a viroporin, and influences inflammatory processes by boosting the activity of the NLRP3 inflammasome, leading to IL-1beta overproduction and development of immunopathology in the host (Nieto-Torres et al. 2015).

## 7 Coronavirus-Induced Deregulation of the Cell Cycle

The cell cycle is a series of highly regulated events that leads to cell division. The process can be divided into four distinct phases: G1, S, G2, and M. Cell cycle regulation is critical for cell survival, as well as the prevention of uncontrolled cell division. The molecular mechanisms that control the cell cycle are ordered, directional, and controlled by cyclin-dependent protein kinases (CDKs). Cell cycle progression requires activation of different CDKs by, e.g., cyclin regulatory subunits. To reach the stage of DNA replication, CDK/cyclin complexes

phosphorylate, and thereby activate or inactivate, their target proteins to coordinate progression towards the next phase of the cell cycle (Nigg 1995).

Like many other viruses [reviewed in (Bagga and Bouchard 2014)], coronaviruses have been shown to extensively manipulate and arrest cell cycle progression, to benefit from the physiological state of cells arrested in that specific phase. For example, IBV-infected cells were shown to go into cell cycle arrest in the S phase, by activating the cellular DNA damage response (Xu et al. 2011). This is beneficial to virus replication since factors that are normally needed for DNA replication and are upregulated in the S phase, can now be recruited to the cytoplasm by the virus. For example, DDX1, a cellular RNA helicase of the DEXD/H family, interacts with coronavirus nsp14 (Xu et al. 2010) and was reported to be hijacked by coronaviruses to enhance their replication. DDX1 also interacts with the IBV N protein (Emmott et al. 2013) and facilitates, in complex with the phosphorylated form of the MHV-JHM N protein, the balanced synthesis of sg mRNAs and the genomic RNA (Wu et al. 2014).

Bhardwaj et al. have shown that coronavirus nsp15 interacts with and inhibits retinoblastoma protein (pRb), a tumor suppressor protein. This results in the enhanced expression of genes that are normally repressed by pRb and in an increased proportion of cells entering the S phase of the cell cycle (Bhardwaj et al. 2012). Similar effects have been observed in MHV-infected cells, which showed decreased hyperphosphorylation of pRb, an event that is necessary for the progression from G1 to S phase (Chen and Makino 2004; Chen et al. 2004). Yuan and colleagues showed that overexpression of the SARS-CoV 3a protein also leads to G1 arrest and inhibition of cell proliferation (Yuan et al. 2007).

The cyclin-dependent kinase 6 (CDK6) is downregulated upon MHV infection and seems to play a role in a virus-induced cell cycle arrest in the G0/G1 phase that promotes virus replication (Chen and Makino 2004). Similar observations have been made for SARS-CoV, with (overexpression of) the N protein limiting cell cycle progression by reducing CDK4 and CDK6 kinase activity (Surjit et al. 2006). CDK6 is a kinase involved in cell cycle progression from G1 to S phase (Jimenez-Guardeno et al. 2014) and depletion of CDK6 results in G1 phase cell cycle arrest. A host kinome-directed siRNA screen confirmed the antiviral role of CDK6 in SARS-CoV infection, as replication was enhanced in cells depleted for CDK6 (de Wilde et al. 2015).

Several coronaviruses induce activation of p53 to mediate cell cycle arrest in the S or G2/M phase. For example, TGEV N protein activates p53 which leads to accumulation of cell cycle-related kinases, like cdc-2 and cyclin B1. Synchronisation of cells in the S or G2/M phase favors TGEV RNA and virus production (Ding et al. 2013, 2014) and IBV replication (Dove et al. 2006). For MHV nsp1 a similar mechanism has been proposed: nsp1 activates p53, leading to increased p21 and decreased CDK2-cyclin E levels. This ultimately leads to hyperphosphorylation of pRb and G1 cell cycle arrest (Chen et al. 2004). In contrast, SARS-CoV infection leads to reduction of p53 expression levels. To counteract the inhibitory effect of p53, its expression is reduced by a mechanism that involves stabilization of the E3 ligase RCHY1 by SARS-CoV nsp3. RCHY1

mediates the ubiquitination and degradation of p53. However, the exact role of this mechanism in cell cycle regulation, viral replication and/or pathogenesis remains unclear (Ma-Lauer et al. 2016). Nevertheless, in cells that lack p53 expression, SARS-CoV replication was significantly enhanced (Ma-Lauer et al. 2016). Degradation of p53 by the SARS-CoV or HCoV-NL63 PL2<sup>pro</sup> is another mechanism to counteract the antiviral effect of p53. The deubiquitinating activity of PL2<sup>pro</sup> promotes the degradation of p53, thereby lowering the p53-mediated antiviral immune response (Yuan et al. 2015).

In conclusion, coronaviruses appear to favor a specific stage in the cell cycle for their replication. This stage can differ per coronavirus and may even differ per cell type. A wide variety of coronavirus proteins have been implicated in inducing cell cycle arrest, but it must be noted that many studies involved overexpression of individual viral proteins and therefore should be interpreted with caution since their expression levels are likely different than during virus infection. When expressed outside the context of virus replication, these proteins may also behave differently (*e.g.*, due to lack of viral interaction partners) and/or localize differently (*e.g.*, in the absence of virus-induced membrane structures), which might lead to less meaningful observations.

## 8 The Role of Cyclophilins in Coronavirus Replication

Cyclosporin A (CsA) is a well-known immunosuppressant that binds to cellular cyclophilins (CyPs), yielding a Cyp-CsA complex that inhibits calcineurin activity. This in turn prevents dephosphorylation and translocation of nuclear factor of activated T cells (NF-AT) from the cytosol into the nucleus, which prevents the transcription of immune genes, such as IL-2 [reviewed in (Tanaka et al. 2013; Davis et al. 2010; Barik 2006)]. Thus far, 17 CyPs have been identified, 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 (Barik 2006; Davis et al. 2010) that facilitate protein folding. CyPs are involved in various signaling pathways [reviewed in (Barik 2006)], apoptosis (Schinzel et al. 2005), and RNA splicing (Teigelkamp et al. 1998; Horowitz et al. 2002).

Cyclophilins, and in particular the cytosolic CypA and the ER-associated CypB, have been implicated in the replicative cycle of many RNA viruses as essential host components [reviewed in (Baugh and Gallay 2012)]. For example, (i) CyPs are essential in the remodeling of cellular membranes into HCV replication organelles, (ii) CypA aids in HCV polyprotein processing, (iii) HIV-1 capsids are stabilized by low levels of CypA to ensure entrance into the nucleus before the virion could be destabilized in the cytosol [(Hopkins and Gallay 2015), and references herein]. The use of CsA analogs, like Alisporivir (Paeshuyse et al. 2006), that lack the immunosuppressive properties of the parental compound, has been explored in clinical trials for the treatment of chronic HCV infection, again illustrating the prominent role of CyPs in HCV replication and the druggability of CyPs (Flisiak et al. 2012).

Along the same lines, the inhibition of coronavirus replication by Cyp inhibitors like CsA and Alisporivir suggested important roles for Cyps. In cell culture infection models, low-micromolar concentrations of CsA or Alisporivir inhibit a variety of coronaviruses, including SARS-CoV, HCoV-229E, MHV, HCoV-NL63, and FCoV (Pfefferle et al. 2011; de Wilde et al. 2011; Tanaka et al. 2012; Carbajo-Lozoya et al. 2012; de Wilde et al. 2017). The mitochondrial CypD is part of the mitochondrial permeabilization transition pore and involved in caspase-independent apoptosis induced by porcine epidemic diarrhea virus (PEDV) and HCoV-NL63 (Favreau et al. 2012; Kim and Lee 2014), an event that is inhibited by CsA. However, most studies on the presumed role of Cyps in coronavirus replication have focused on CypA. Initially, CypA was identified as an interaction partner of the SARS-CoV N protein (Luo et al. 2004) and by mass spectrometry it was also detected in purified SARS-CoV virions (Neuman et al. 2008). Yeast two-hybrid experiments identified multiple Cyps (CypA, CypB, CypH, and CypG) and related PPIases (FK506-binding protein 1A and 1B) as potential binding partners of SARS-CoV nsp1 (Pfefferle et al. 2011). In addition, in Caco-2 or Huh7 cells, shRNA-mediated knockdown of CypA expression to less than 3% of the normal levels was reported to near-completely block HCoV-NL63 replication and reduce HCoV-229E replication by >1 log (Carbajo-Lozoya et al. 2014; von Brunn et al. 2015). Recently, by using PPIA or PPIB knockout rather than knockdown cells, it was shown that FCoV depends on both CypA and CypB expression (>95% reduction in FCoV-infected CypA or CypB-KO cells). PPIase-deficient mutants, expressed in cells that also contained endogenous CypA and CypB, marginally reduced FCoV infection (two- to fivefold reduction in FCoV RNA compared to cells that express wt CypA or wt CypB only) (Tanaka et al. 2016). Furthermore, the work of von Brunn and colleagues suggested that HCoV-229E replication was reduced by various CypA single-nucleotide polymorphisms that affect the protein's stability and function (von Brunn et al. 2015). Although these results suggested a role for CypA in coronavirus replication, the siRNA-mediated depletion of CypA and CypB did not affect the replication of SARS-CoV (de Wilde et al. 2011), despite the fact that the same knockdown did affect the replication of the distantly related arterivirus EAV (de Wilde et al. 2013a). The differences reported using either knockout or knockdown of CypA with different efficiencies (100%, >97%, and ~75% for CypA knockout cells, and CypA shRNA- or siRNA-mediated knockdown, respectively) suggest that low CypA expression levels may suffice to support efficient coronavirus replication and that the (near-)complete depletion of CypA may be needed to inhibit virus replication.

The major role of Cyps in cellular signaling is in the NF-AT signaling pathway. Pfefferle et al. described that SARS-CoV activates the NF-AT signaling pathway (Pfefferle et al. 2011) and the replication of various coronaviruses, including SARS-CoV, HCoV-229E, and HCoV-NL63, is inhibited by the drug FK-506, which—like CsA—also blocks NF-AT signaling (Carbajo-Lozoya et al. 2012). Feline coronavirus replication seemed not to depend on a functional NF-AT signaling pathway since it was blocked by CsA concentrations that did not affect NF-AT signaling in fcwf-4 cells (Tanaka et al. 2012).

In conclusion, further studies, are needed to dissect the precise role of Cyps in coronavirus replication, with special attention for the (remaining) levels of CypA expression, and the relevance of NF-AT signaling following coronavirus infection. Such experiments should include the production and use of knockout cells for one or multiple Cyps or other members of this protein family. Although Cyp inhibitors have been shown to be potent anti-coronavirus drugs in cell culture, a first attempt to validate this effect for Alisporivir in a SARS-CoV animal model was unsuccessful (de Wilde et al. 2017). Therefore, although Cyp inhibitors may help to understand the role of host factors in coronavirus replication, they are at this moment less promising as host-directed therapeutics for the treatment of coronavirus infections.

## 9 Systems Biology Approaches to Identifying Host Factors in Coronavirus Replication

The application of systems biology approaches in virology has provided a wealth of information on the role of individual proteins and cellular pathways in the replication of RNA viruses. This relatively young, interdisciplinary field focuses on the complexity of the virus–host interactions that occur within the cell or even the whole organism. The aim is to provide an unbiased perspective, by applying techniques like transcriptomics, metabolomics, proteomics, and functional genomics to the infected system as a whole.

For coronaviruses, one of the first systematic studies into the role of host factors concerned an oligonucleotide microarray-based transcriptomic analysis of SARS-CoV-infected peripheral blood mononuclear cells, which revealed the upregulation of the expression of various cytokines, including IL-8 and IL-17, and the activation of macrophages and the coagulation pathway (Ng et al. 2004). A microarray analysis of lung autopsy tissue samples provided more insight into the pathogenesis of and host response to SARS-CoV infection, in particular the inflammatory and cytokine responses involved (Baas et al. 2006). MHV-JHM infected cultures of central nervous system cells showed 126 differentially expressed transcripts, the majority of which were related to intracellular regulation of innate immunity (*e.g.*, NF- $\kappa$ B signaling and genes involved in IFN signaling) (Rempel et al. 2005). Microarray analysis of MHV-A59-infected L cells provided insight into transcriptional changes during infection, including those related to chemokine production, RNA and protein metabolism and apoptosis (Versteeg et al. 2006). Subsequently, a genome-wide microarray analysis of MHV-infected LR7 cells revealed the downregulation of a large number of mRNAs, including many encoding proteins involved in translation, implying that the host translational shut-off that occurs in MHV-infected cells is due to a stress response and concomitant mRNA decay (Raaben et al. 2007). There is not necessarily a direct correlation between changes in mRNA levels and protein abundance in the cell.

Therefore, microarray data should be interpreted with caution and need to be validated with follow-up experiments including the direct analysis of changes in cellular protein levels.

Another approach to obtain more insight into coronavirus–host interactions is to systematically map the cellular interactome of individual coronavirus proteins. A yeast two-hybrid (Y2H) screen of this type identified subunits (BTF3 and ATF5) of the RNA polymerase complex and a subunit of cytochrome oxidase II (NADH 4L) as interaction partners of SARS-CoV nsp10 (Li et al. 2005a). The authors suggested the latter interaction to contribute to the SARS-CoV-induced cytopathic effect, but the interaction and its relevance for virus-induced cell death still awaits confirmation in the context of SARS-CoV-infected cells, as the rather artificial Y2H system is known to frequently yield false-positive hits. Indeed, another Y2H study with the SARS-CoV helicase (nsp13) demonstrated that out of the seven primary hits only one, DDX5, could be validated by independent methods as a true interactor of the helicase nsp13 (Chen et al. 2009). The functional significance of the interaction between nsp13 and the multifunctional cellular helicase DDX5, and whether the interaction is direct or mediated through RNA, remains to be determined. Using a similar Y2H screening approach, Xu and colleagues found that DDX1 bound to SARS-CoV and IBV nsp14, an interaction that enhanced IBV replication. The presence of one or more cellular helicases in the viral RTC supports their importance in virus replication. Indeed a later study reported that DDX1, regulated by the GSK-3-mediated phosphorylation of the N protein, is involved in the regulation of sg mRNA synthesis (Wu et al. 2014). A very comprehensive systematic analysis of the SARS-CoV-host interactome was performed by Pfefferle et al. using a high-throughput genome-wide Y2H screen with all 14 SARS-CoV ORFs and fragments thereof (Pfefferle et al. 2011). Network analysis revealed particularly striking interactions between nsp1 and the members of the immunophilin family, including CypA (see Chap. 8). Furthermore, the cellular E3 ubiquitin ligase RCHY1 was shown to interact with the SARS-CoV nsp3 SUD domain and might be involved in the downregulation of the antiviral factor p53 (Ma-Lauer et al. 2016).

Stable isotope labeling with amino acids in cell culture (SILAC) is a mass spectrometry-based proteomics technique to determine differences in protein abundance between samples from two different experimental conditions, *e.g.*, comparing virus-infected with uninfected cells. The first SILAC-based quantitative proteomics study of infected cells demonstrated the upregulation of NF- $\kappa$ B and AP-1 dependent pathways during IBV infection (Emmott et al. 2010). A combination of SILAC on 293T cells that express the IBV N protein, pull-down and mass spectrometry was used to map the cellular interactome of the IBV N protein, leading to the identification of 142 cellular proteins as potential binding partners (Emmott et al. 2013). Many of these proteins are interacting with RNA, *e.g.*, ribosomal and nucleolar proteins, helicases, and hnRNPs (Emmott et al. 2013) and therefore likely bind the IBV N protein indirectly. Nevertheless, detailed validation and mechanistic follow-up studies confirmed the functional importance of several of the identified binding partners for IBV replication (Emmott et al. 2013).

The Baric laboratory used a systems genetics approach using the Collaborative Cross mouse panel to gain insight into the host loci that affect the outcome of SARS-CoV infection (Gralinski et al. 2015). This study—among other findings—identified the ubiquitin E3 ligase Trim55 as an important determinant of disease severity through its role in vascular cuffing and inflammation. A study by Selinger et al. (2014) documented differences in immune and inflammatory responses in MERS patients, which may codetermine the outcome of the infection and likely result from both differences in host response (genetic make-up) and MERS-CoV strain-specific properties. To better understand the molecular basis of the different immune and inflammatory responses to two different MERS-CoV isolates, a comparative transcriptome analysis was done on human airway cells infected with MERS-CoV strains SA1 and Eng1 (Selinger et al. 2014). This study suggested that differences in genome replication and/or proteins involved in innate immune evasion (PL<sup>pro</sup> and ORF4a) were responsible for different transcriptional responses, resulting in the differential activation of the STAT3 pathway, which is likely involved in lung inflammation and cellular repair. These effects are mainly seen during later stages of infection, and with the MERS-CoV Eng1 strain triggering a more rapid host response than the SA1 strain.

A SILAC-based quantitative proteomics study that compared the proteome of SARS-CoV replicon-expressing BHK-21 cells with that of control cells identified 43 host proteins whose expression was upregulated and 31 that were downregulated (Zhang et al. 2010). BAG3, a multifunctional regulator of many cellular processes was identified as one of the upregulated proteins and knockdown studies revealed that BAG3 is important for efficient replication of SARS-CoV and a number of other viruses. In addition, many proteins involved in translation and the signaling proteins Cdc42 and RhoA were shown to be downregulated, as discussed in more detail in Sect. 3 (Zhang et al. 2010). A SILAC-based quantitative proteomics study of Golgi-enriched subcellular fractions revealed that upon MHV infection several proteins of the secretory pathway were depleted, while ribosomal proteins were found to be enriched (Vogels et al. 2011). siRNA-mediated knockdown of three of the depleted proteins, C11orf59, GLG1, and sec22b, increased replication or release of infectious progeny, while overexpression of these proteins had the opposite effect. This study highlighted the importance of the secretory pathway in coronavirus replication.

A role for secretory pathway proteins was also confirmed by a proteomic analysis of purified SARS-CoV virions, which identified, besides several viral proteins including nsp2, nsp3, and nsp5, 172 host proteins in virions (Neuman et al. 2008). Several proteins from the COPI pathway were identified, which is in line with the site of virion biogenesis (ERGIC). The role, if any, of most of the identified host proteins in SARS-CoV replication or virion biogenesis remains to be elucidated.

Protein kinases are key regulators in signal transduction, control a wide variety of cellular processes, and have been shown to play important roles in the replicative cycle of many +RNA viruses. A kinome-wide siRNA screen identified a variety of host cell kinases that influence SARS-CoV replication, including 40 ‘proviral’

proteins that promote efficient replication (de Wilde et al. 2015). Among these, proteins involved in the metabolism of complex lipids and the early secretory pathway (COPI-coated vesicles) were found to play an important role. The antiviral effect of PKR was confirmed in this study and CDK6 was identified as a novel antiviral factor. A relatively large number of antiviral hits (90 of 778 factors; ~12% of all factors tested) was identified for SARS-CoV compared to human kinome-directed screens performed with other viruses (Supekova et al. 2008; Lupberger et al. 2011; Moser et al. 2010). This might indicate that, compared to other viruses, SARS-CoV replication is more extensively influenced by cellular factors. Multiple of these factors could be linked to cellular immune responses, like interleukin (IL) signaling, which (IL-6 and -8) was also implicated in controlling coronavirus infection and coronavirus-induced inflammation in other studies (Zhang et al. 2007; Baas et al. 2006). Also several proteins from the p38 MAPK pathway were identified, which had also been implicated in coronavirus replication earlier and regulates IL-6-, IL-8-, and IL-10-mediated pro-inflammatory cytokine signaling (Chang et al. 2004; Zhang et al. 2007; Song et al. 2013). The interaction between coronaviruses and the innate immune response was already discussed in more detail in Chap. 6. A similar, genome-wide, siRNA screen identified host proteins important for the replication of IBV (Wong et al. 2015), including 83 proviral proteins, 30 of which could be mapped to networks that interact with viral proteins. Many of the identified proteins are involved in RNA binding/processing, membrane trafficking and ubiquitin conjugation. The importance of the secretory pathway that was demonstrated by de Wilde et al. (2015) was in line with an earlier study that demonstrated that MHV replication was sensitive to Brefeldin A treatment and dependent on GBF1-mediated ARF1 activation, which appear to be involved in RTC formation (Verheije et al. 2008). Similar results were also obtained for IBV (Wong et al. 2015) and this study also identified an early role in IBV infection for the valosin-containing protein (VCP) which may be involved in the maturation of virus-loaded endosomes. VCP is also important for the early stages of HCoV-229E replication (Wong et al. 2015). An RNAi screen of the druggable genome identified several endocytosis-related proteins that are required for efficient infection of HeLa cells with MHV (Burkard et al. 2014). Subsequent validation and mechanistic studies, demonstrated that—as discussed above—clathrin-mediated endocytosis and trafficking to lysosomes are crucial for MHV fusion and entry, which required the activity of lysosomal proteases. This is different for MERS-CoV, which contains a furin cleavage site upstream of the fusion peptide in the Spike protein, and therefore requires furin activity, but not lysosomal proteases (Burkard et al. 2014).

RNAi screens have revolutionized functional genetics, but major concerns are the possibility of false-positive hits due to off-target effects (including downregulation of multiple transcripts), stimulation of the immune response, or saturation of the RNAi machinery leading to a block in processing of essential cellular (mi) RNAs [reviewed in (Jackson and Linsley 2010)]. Insufficient knockdown of host

factors can lead to false-negative results. Therefore, hits from siRNA screens need to be thoroughly validated, preferably using an independent technical approach. They should be considered as a mere starting point for further analysis rather than providing a definitive list of host factors involved in virus replication. Technological advancements and novel screening approaches, e.g. those based on CRISPR/Cas9-mediated genome editing or haploid genetic screens (Shalem et al. 2014; Carette et al. 2009), will likely lead to the more reliable identification of host factors important for coronavirus replication.

## 10 Concluding Remarks

Insight into coronavirus-host interactions, obtained, e.g., using systematic screening approaches, does not only yield valuable information on the molecular details of the replicative cycle and pathogenesis, but can also be a starting point for the development of antiviral strategies. Virus binding and entry are the first steps of the replication cycle that can be targeted with inhibitors. Several well-known inhibitors of endosomal acidification, like ammonium chloride and the FDA-approved anti-malaria drug chloroquine, have been shown to block entry of coronaviruses (Takano et al. 2013; Keyaerts et al. 2009; Krzystyniak and Dupuy 1984; Payne et al. 1990; Kono et al. 2008), including SARS-CoV and MERS-CoV (Keyaerts et al. 2004; de Wilde et al. 2014). In addition, peptides have been developed that block fusion by interfering with the interaction between the HR1 and HR2 domains of the S protein, preventing the formation of a fusogenic complex or blocking S protein oligomerisation [reviewed in (Du et al. 2009)].

Interferon (IFN) was shown to trigger the innate immune response in coronavirus-infected cells, leading to transcription of many ISGs that have a role in controlling infection (Schoggins and Rice 2011). Treatment with type-I IFNs inhibits coronavirus replication in cell culture (Garlinghouse et al. 1984; Taguchi and Siddell 1985; Haagmans et al. 2004; Paragas et al. 2005; Zheng et al. 2004; de Wilde et al. 2013b) and, for example, protected type-I pneumocytes against SARS-CoV infection in macaques (Haagmans et al. 2004). Despite the potency of IFN as an antiviral agent, its side effects like fatigue, malaise, apathy, and cognitive changes (Dusheiko 1997) emphasize the need for developing IFN-free therapeutic strategies. Besides inhibitors directed at viral enzymes (Kim et al. 2016), such therapeutic strategies could also involve host-directed approaches, based on the knowledge obtained on coronavirus–host interactions. The host-directed approach might lower the chance of development of antiviral resistance and could yield a broad-spectrum therapeutic strategy to treat infections with currently problematic coronaviruses and new variants that will undoubtedly emerge in the future.

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