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Feline infectious peritonitis virus accessory proteins and type I interferon are fighting for dominance

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List of abbreviations

2-5A	2’-5’-phosphodiester bounded oligoadenylates
A	adenosine
AA	amino acid
ADAR	adenosine deaminase acting on RNA
ADCC	antibody-dependent cell-mediated cytotoxicity
ADCML	antibody-dependent complement mediated lysis
ADEI	antibody-dependent enhancement of infectivity
AP-1	activation protein-1
APC	antigen-presenting cell
APL	acute promyelocytic leukaemia
APOBEC	apolipoprotein B mRNA editing enzyme-catalytic polypeptide
BST-2	bone marrow stromal antigen 2 (=tetherin)
BMM	bone-marrow derived macrophage
C	cytidine
CARD	caspase activation and recruitment domains
CCV	canine coronavirus
CD	cluster of differentiation
CoV	coronavirus
DC	dendritic cell
DC-SIGN	dendritic cell-specific intercellular adhesion molecule grabbing non-integrin
DMV	double membrane vesicle
dsRNA	double stranded RNA
E	small envelope protein
eIF2a	eukaryotic translation initiation factor 2 alpha
ER	endoplasmic reticulum
ERGIC	ER to Golgi intermediate compartment
ERK	extracellular signal-regulated kinase
fAPN	feline aminopeptidase N
FCoV	feline coronavirus
FECV	feline enteric coronavirus
FIP	feline infectious peritonitis
FIPV	feline infectious peritonitis virus

FIV	feline immunodeficiency virus
G	guanine
GADD34	growth arrest and DNA damage-inducible protein
GTPase	guanosine triphosphatase
HBV	hepatitis B virus
HCMV	human cytomegalovirus
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HSV	herpes simplex virus
I	inosine
IBV	infectious bronchitis virus
ICAM	intercellular adhesion molecule
IFIT	interferon-induced proteins with tetratricopeptide repeats
IFITM	interferon-induced transmembrane
IFN	interferon
IFNAR	type I interferon receptor
Ig	immunoglobulin
IκB	inhibitor kappa B
IKK	IκB kinase
IL	interleukin
IRF	interferon regulatory factor
ISG	interferon-stimulated gene
ISGF3	interferon-stimulated transcription factor-3
ISRE	interferon-stimulated response element
JAK	Janus kinase
JNK	c-Jun N terminal kinase
kb	kilobase
kDa	kilodalton
KPNA2	karyopherin alpha 2
LGP2	laboratory of genetics and physiology 2
LN	mesenteric lymph node
LPS	lipopolysaccharide
M	membrane protein

Mac-1	macrophage-1 antigen
MAPK	mitogen-activated protein kinase
MAVS	mitochondrial antiviral signalling protein
MDA5	melanoma differentiation-associated gene 5
MERS-CoV	Middle East respiratory syndrome coronavirus
MHC	Major histocompatibility complex
MHV	mouse hepatitis virus
Mx	myxovirus resistance protein
MyD88	myeloid differentiation primary response protein 88
N	nucleocapsid protein
NF-κB	nuclear factor kappa B
NK	natural killer
NLS	nuclear-localisation signal
nsp	non-structural protein
OAS	2’-5’-oligoadenylate synthetase
ORF	open reading frame
PAMP	pathogen-associated molecular patterns
PKR	interferon-induced dsRNA dependent protein kinase
PLpro	papain-like protease
PML NB	promyelocytic leukaemia nuclear body
pp	polyprotein
PRR	pattern recognition receptor
rER	rough endoplasmatic reticulum
RIG-I	retinoic acid inducible gene-I
RING	really interesting new gene
RLR	RIG-I like receptor
RNA	ribonucleic acid
RNaseL	endoribonuclease L
RTC	replication-transcription complex
S	spike protein
SARS-CoV	severe acute respiratory syndrome coronavirus
sg	subgenomic
ssRNA	single stranded RNA

STAT	signal transducer activator of transcription
STING	stimulator of interferon genes
SUMO	small ubiquitin modifier
T	thymidine
TANK	TRAF family member-associated NF-kappa-B activator
TBK	TANK-binding kinase
TF	transcription factor
TGEV	transmissible gastro-enteritis virus
TIR	toll/interleukin-1 receptor
TLR	toll-like receptor
TNF-α	tumor necrosis factor alpha
TRAF	tumor necrosis factor receptor-associated factor
TRIF	TIR domain-containing adapter inducing IFN-β
TRIM	tripartite motif
TRS	transcription regulation sequence
TYK	tyrosine kinase
U	uridine
UPR	unfolded protein response
Viperin	virus inhibitory protein, ER-associated, interferon-inducible
VV	vaccinia virus

Chapter 1

Introduction

1.1 Feline Coronaviruses

1.1.1 Historical background: virus discovery, pathotypes and serotypes

Fifty years ago, a new "highly important disease of cats" was identified by Holzworth and it was called feline infectious peritonitis (FIP) (Holzworth, 1963). It took another five years to prove that it was caused by an infectious agent (Zook et al., 1968), which was categorized as a member of the family *Coronaviridae* by Ward in 1970 (Ward, 1970). In the following decade, the morphological and physical characteristics of this coronavirus were further analysed and fully virulent feline infectious peritonitis virus (FIPV) could be propagated *in vitro* (Black, 1980; O'Reilly et al., 1979; Pedersen, 1976; Pedersen et al., 1978). Cats infected with the highly lethal FIPV, succumb as a direct consequence of the typical granulomatous lesions in various organs, vasculitis and organ failure (Olsen, 1993). In 1981, a second feline coronavirus (FCoV) was described, feline enteric coronavirus (FECV), which is not inducing severe clinical signs in infected cats (Pedersen et al., 1981a). Infection with FECV is until now very common in shelter cats and other densely housed feline populations.

It is still not fully elucidated if FIPV emerges as a virulent variant of FECV or whether they are two different virus strains circulating in the feline population (Brown et al., 2009; Vennema, 1999). Due to the close genetic similarity between FECV and FIPV, and the contradiction between the low FIP incidence and the high FCoV seropositivity, the "internal mutation theory" is the generally accepted hypothesis for the FIPV origin (Vennema et al., 1998). Numerous publications support that FIPV, unlike FECV, is not transmitted from cat to cat but arises from FECV by mutation within an individual cat (Foley et al., 1997; Herrewegh et al., 1995; Poland et al., 1996; Rottier et al., 2005; Vennema et al., 1998). Recently, two of the potential mutational causes of FIPV were described by Chang et al., being two amino acid changes in the fusion peptide of the spike protein (Chang et al., 2012). Also deletions/mutations in the accessory protein 3c have been linked to FIPV pathotypes (Chang et al., 2010; Pedersen et al., 2012). Next to viral factors, susceptibility to FIP also depends on individual host factors such as age, sex and genetic predisposition (Foley and Pedersen, 1996).

The extra subdivision of the pathotypes into two serotypes (type I and II), renders the FCoV story even more complex. Type II feline coronaviruses originate from a double recombination between type I feline coronavirus and canine coronavirus (CCV), resulting in the exchange of ORF3 and the neighbouring spike sequence (Herrewegh et al., 1998). Consequently, the subdivision is based on the *in vitro* neutralisation using monoclonal

antibodies against the spike protein. Type I feline coronaviruses have a higher prevalence in the field (Hohdatsu et al., 1992; Vennema, 1999). However, most experiments have been done with type II viruses because type I viruses are difficult to grow *in vitro* (Pedersen, 1987). Recently, Desmarets et al. figured out a way to overcome this important obstacle for type I FECV strains by establishing feline intestinal epithelial cell cultures which can be used to grow and study enteric FCoV field strains (Desmarets et al., 2013).

1.1.2 Classification

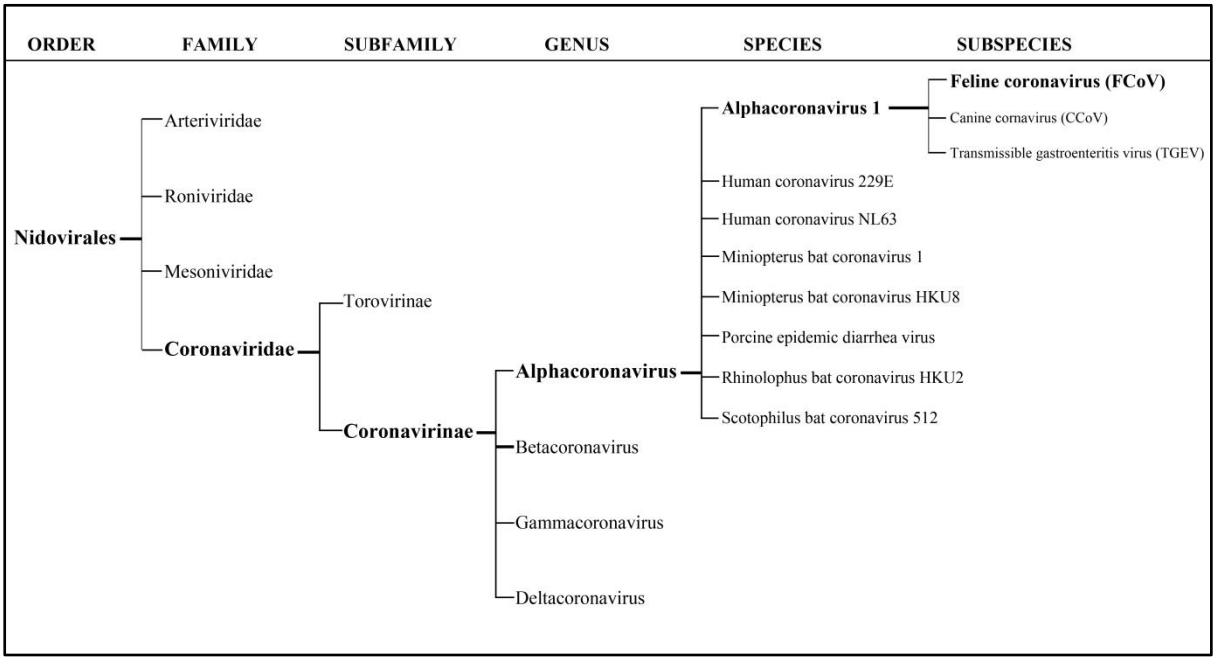


Figure 1.1: Classification tree of *alphacoronavirus* genus (Adapted from King et al., 2012)

Feline coronaviruses belong to the *Coronaviridae*, identified as a family of large enveloped viruses (60-200nm) containing a single-stranded RNA genome of positive polarity (Siddell, 1995). They rank among the largest known RNA genomes, with sizes up to 31 kilobases (kb). Together with the family of the *Arteriviridae*, *Roniviridae* and *Mesoniviridae*, they form the order of the *Nidovirales*. The unique composition of the replicase gene, the polycistronic genome organisation and remarkable replication strategy discriminates nidoviruses from other RNA viruses. In all nidoviruses, the replicase gene is expressed from two huge overlapping open reading frames (ORFs) (ORF1a and ORF1b), which occupy more than two-thirds of the genome starting from the 5' end. The ORFs located downstream of the ORF1a/b are expressed from a nested set of subgenomic RNAs, which inspired the name of the *Nidovirales* order (from the Latin *nidus*, nest) (De Vries et al., 1997; Gorbalenya et al., 2006). *Alpha-*, *beta-*, *gamma-* and *deltacoronaviruses* are four genera in the subfamily

Coronavirinae of the family *Coronaviridae*, which can be distinguished based on rooted phylogeny and unique replicase domain sequences (King et al., 2012). Feline coronaviruses belong to the species of the *Alphacoronavirus 1*, within the genus of the *Alphacoronavirus* (Figure 1.1).

1.1.3 Virion architecture

Coronaviruses have spherical virions characterized by an external phospholipid bilayer (envelope) with associated structural proteins (spike, membrane and small envelope protein) that encloses the loosely wound helical nucleocapsid structure (Figure 1.2A). They gain the typical "crown" like appearance by electron microscopy due to the large envelope protrusions of spike proteins (peplomers) (De Vries et al., 1997; Gorbalenya et al., 2006; Siddell, 1995).

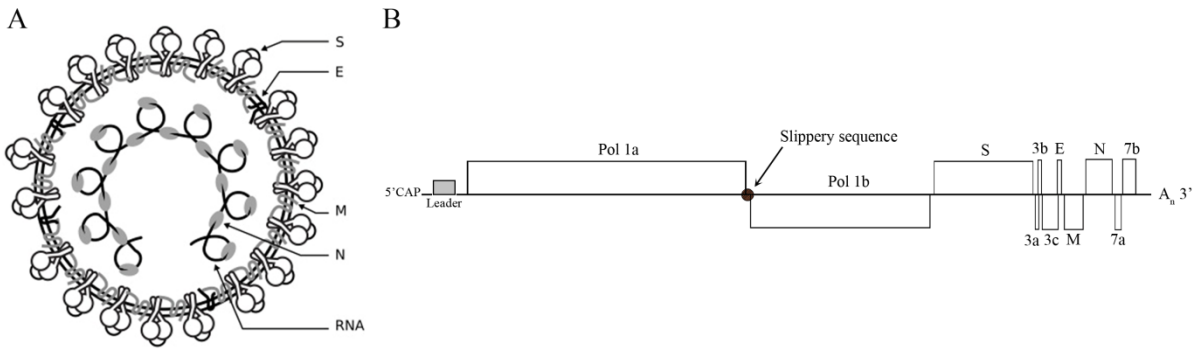


Figure 1.2: A) Schematic overview of a feline coronavirus particle with RNA, envelope (E), spike (S), membrane (M) and nucleocapsid (N) proteins (Dewerchin, 2008). **B) Schematic representation of the genomic organization of FCoVs**

Viral Genome

Coronaviral genome RNA is single-stranded, 3'polyadenylated and 5' capped (Figure 1.2B). The first two-thirds of the genome is taken up by two overlapping ORFs; ORF1a and ORF1b. The small overlap contains a -1 ribosomal frameshift signal which allows a read-through for a fraction of ribosomes by a pseudoknot structure, resulting in further translation of ORF1b. The polyproteins (pp1a and pp1ab) encoded by these ORFs, are autoproteolytic cleaved by two viral proteases, a papain-like protease (nsp3) and a 3C-like protease (nsp5), which are within pp1a and pp1ab (Thiel et al., 2003). This yields 16 nonstructural proteins (nsps) that assemble with other viral and probably cellular proteins to a membrane-bound replication-transcription complex (RTC) in the cytoplasm of the cell (Gosert et al., 2002; Sawicki et al.,

2007; Ziebuhr et al., 2000). Among others, the RNA helicase activity (nsp13), exoribonuclease activity (nsp14), endoribonuclease activity (nsp15), methyltransferase activity (nsp16) and viral RNA-dependent RNA polymerase (nsp12) are functions shown to be important for the replication and transcription potential of the RTC (Cornillez-Ty et al., 2009). Recently, it was also proven by Denison et al. that the nsp14-exoribonuclease is controlling the mutation rate of coronaviruses, by acting as an important mediator of a RNA proofreading machinery, which was not yet identified for other RNA viruses before (Denison et al., 2011). The ORFs encoding structural proteins are located downstream of ORF1b, being ORF2 (spike, S), 4 (small envelope, E), 5 (membrane, M) and 6 (nucleocapsid, N). Next to these ORFs, feline coronaviruses also possess 5 accessory or group-specific proteins that are encoded from 2 ORF clusters, being ORF3 (encoding 3a, 3b and 3c protein) and ORF7 (encoding 7a and 7b). The ORFs located in the 3'-part of the genome are expressed from a nested set of subgenomic RNAs, which are both 3' and 5' coterminal. Although these mRNAs are structurally polycistronic, most of them are functionally monocistronic, as only the most 5' located ORF is translated (Lai and Cavanagh, 1997). ORF3 and ORF7 make the exception because they contain more translationally active areas. For these ORFs ribosomal leaky scanning is proposed to encode the different proteins from one and the same ORF (Schaecher et al., 2007).

Viral proteins

The nucleocapsid protein (N) is basic with an acidic C-terminus and has a molecular weight of about 45-63 kilodalton (kDa). It consists of 8 to 10% serine residues, which are typically phosphorylated , anticipating in the association with intracellular membranes (Siddell, 1995; Spaan et al., 1988; Stohlman et al., 1983). Due to its basic character, it can bind to the viral RNA, whereby the genome is encapsulated in a stable helical nucleocapsid structure (Parker and Masters, 1990). Interactions between N and M protein encourage the incorporation of this nucleocapsid structure into newly formed virions (Narayanan et al., 2000). Coronavirus N protein has been implicated in virus RNA synthesis, considering its specific RNA binding capacity, its partial RTC localisation and its requirement for efficient replication (Sawicki et al., 2007). Moreover, a fraction is also localised in the nucleoli, from where it might ensure maximal viral mRNA translation by delaying the cell cycle in the interphase and sequestering ribosomes to viral mRNA (Chen et al., 2002). Recently, N proteins of mouse hepatitis virus (MHV) A59 and severe acute respiratory syndrome coronavirus (SARS-CoV) have been implicated in circumventing the interferon (IFN) immune response by interfering with IFN

production or IFN-stimulated gene (ISG) activity, respectively, through binding to dsRNA (Kopecky-Bromberg et al., 2007; Lu et al., 2011b; Ye et al., 2007).

The membrane protein (M) is the major component of the viral envelope, with a molecular weight of 20-38 kDa. The hydrophobic nature of a large region of the protein dictates its typical topology (three membrane spanning domains with only small parts protruding out- and inside the virion) and its intracellular transport (synthesized on membrane-bound ribosomes, wherefrom it passages through the ER and Golgi complex to finally reach the outside of the cell, embedded in the envelope of the virions) (Armstrong et al., 1984; Siddell, 1995; Spaan et al., 1988). It is invariable glycosylated, which provides the virion with a hydrophilic cover on his outer surface. The glycosoylation state determines the interferogenic capacity of the M protein, as M proteins with N-linked sugars are far better type I interferon inducers than O-linked M proteins (de Haan et al., 2003). M protein is the only viral glycoprotein required for virus budding at the ER-Golgi intermediate compartment (ERGIC). In an infected cell, it interacts with the nucleocapsids by its cytoplasmic domain and forms heteromultimeric complexes with the spike protein, thereby ensuring their encapsulation in newly formed virions (Opstelten et al., 1995; Rottier and Rose, 1987; Siddell, 1995). It was also demonstrated that the M protein determines one of the immune evasion processes employed by FCoVs, as the M proteins resident in the cell membrane are internalized upon antibody addition, reducing efficacy of antibody-mediated cell lysis (Dewerchin et al., 2006).

The spike protein (S) is a large type I membrane glycoprotein (180-210 kDa) that possesses a short C-terminal sequence inside the virion, preceded by a single transmembrane domain and a large N-terminus with plenty potential N-glycosylation sites facing the virion exterior (Bosch et al., 2005; Delmas and Laude, 1990; Masters, 2006; Siddell, 1995). The S glycoprotein appears as a trimeric spike that is the major determinant of cell tropism by mediating coronavirus attachment and virus-induced membrane fusion by the S1 and core S2 part, respectively (Beniac et al., 2006; Beniac et al., 2007). This fusogenic capacity results in both virus-cell fusion, which results in genome release in the cytoplasm, and cell-cell fusion, when being transported to the plasma membrane, characterized by syncytium formation (De Groot et al., 1989; Delmas and Laude, 1990). The S protein is the major inducer of neutralizing antibodies against FCoVs, mainly due to epitopes on the S1 part, and is important in several cell-mediated immune responses (Delmas and Laude, 1990; Dewerchin et al., 2006). Finally, variations within S of FCoV may be linked to the different pathotypes (Chang et al., 2012; Fiscus et al., 1987; Licitra et al., 2013; Rottier et al., 2005)

The small envelope protein (E) is present in low numbers in the virion as an 10 kDa membrane protein, spanning the lipid bilayer twice, with only a small part of the N-terminus extending inside the virion (Maeda et al., 2001). It has been proposed to facilitate virus release from infected cells by altering the cellular permeability by forming membrane channels (Liao et al., 2006; Madan et al., 2005; Wilson et al., 2006; Wilson et al., 2004). Furthermore, it is essential for virus maturation as it captures other membrane proteins at the budding site, ensuring the creation of new infective virus particles (Kuo and Masters, 2003; Lim and Liu, 2001; Lim et al., 2001).

The accessory proteins are subgroup-specific proteins which expression is preserved during evolution, providing the virus additional selective advantages. However, the study of these proteins encounters potential difficulties, due to the low molecular weight, low expression levels and/or non-structural properties. Moreover, very little homology can be found between different virus species. Many accessory proteins are now know to be dispensable for coronavirus growth in cell culture, but they seem to be important for *in vivo* replication, as deletion of the accessory ORF clusters resulted in attenuated viruses (de Haan et al., 2002; Haijema et al., 2004; Ortego et al., 2003; Zhang et al., 2007). This stresses their importance in virulence and virus-host interactions but unfortunately, the functional information about the FCoV accessory proteins remains scarce.

FCoVs have three accessory genes (3a, 3b and 3c), clustered in one ORF, called ORF3, located between the S and E genes and two others (7a and 7b) downstream of the N gene, clustered in ORF7 (Gorbalenya et al., 2006).

Proteins 3a and 3b are 72 amino acids (AA) long and are most probably expressed as soluble proteins. Their sequences are well conserved within the two pathotypes (Volker, 2007). Hydrophobicity analysis predicts that protein 3c (238 AA) is a class III triple-membrane spanning protein, suggesting a topology that is quite similar to the one of the M protein of FCoVs and the protein 3a of SARS-CoV (Narayanan et al., 2008b; Oostra et al., 2006). The protein 3c has been linked to development of FIPV strains out of FECV, due to the frequent appearance of truncated 3c genes in FIP isolates (Chang et al., 2010; Pedersen et al., 2012) and the inefficient *in vitro* replication in feline monocytes of FIPV type II with intact ORF3 (Balint et al., 2012).

Protein 7a, a small hydrophobic protein of 101 AA (~10kDa) with a N-terminal cleavable signal sequence and a C-terminal transmembrane domain, is more or less well conserved among FCoVs (Volker, 2007). It has a 72% homology with transmissible gastro-enteritis

virus (TGEV) protein 7 which has been demonstrated to interfere with host antiviral responses (see part 1.3.4) (Cruz et al., 2011).

Most practical analysis has been done with protein 7b, which is a soluble glycoprotein of ~24 kDa (206 AA). A lot of single amino acid polymorphisms are present between FCoV strains. Antibodies against protein 7b, found in serum of FECV-seropositive and FIP cats, imply expression of this protein during natural infections (Kennedy et al., 2008). It contains a KDEL-like endoplasmic reticulum (ER) retention signal (=KTEL) at its C-terminus (Vennema et al., 1992). Protein 7b is initially retained in the ER, whereupon it is secreted from infected cells. As 7b is released extracellular, it might function as a virokine which acts as a competitive inhibitor of host cytokine(s) to modulate the host immune or inflammatory reaction (Herrewegh et al., 1995; Rottier, 1999) or it might be responsible for the observed induction of T-cell depletion during FIPV pathogenesis (see part 1.1.6) (Haagmans et al., 1996). Again the precise function remains enigmatic.

1.1.4 Replication cycle

Entry

Target cells of FCoVs are characterized by the presence of a specific receptor on the plasma membrane that allows the virus particles to initiate entry of the cell. Two potent receptors have been described for FCoVs, being feline aminopeptidase N (fAPN), a cell-surface type II metalloprotease, and feline dendritic cell (DC)-specific intercellular adhesion molecule (ICAM) grabbing non-integrin (DC-SIGN) (Regan and Whittaker, 2008; Tresnan and Holmes, 1998; Tresnan et al., 1996). Still, these two receptors are not the only virus binding receptors of the *in vivo* target cell, the monocyte, as binding is only for about 60% inhibited by blocking these molecules (Van Hamme et al., 2011). So, other, still unidentified, receptor(s) may be important for virus entry. Moreover, a clear difference in virus binding is described for type I and II strains (Dye et al., 2007; Hohdatsu et al., 1998). Type I uses fDC-SIGN and unidentified receptor(s) to mediate virus binding and all internalized particles depend on fDC-SIGN for infection of monocytes. Cell binding of type II FCoVs relies on both fAPN and unknown receptor(s) and is thus independent of fDC-SIGN. Still, the presence of both fAPN and fDC-SIGN is needed to establish complete infection (Van Hamme et al., 2011). After receptor binding, FIPV particles are efficiently internalized by a clathrin- and calveolin- independent endocytosis, detained in intracellular vesicles (Figure 1.4 step 1-2) (Van Hamme et al., 2007; Van Hamme et al., 2008). In only a restricted subpopulation of monocytes, the FIPV genome is released in the cytosol (Figure 1.4 step 3).

It is not yet fully elucidated what the possible triggers for virus uncoating are. Both low pH and cleavage by proteases, as well as receptor-induced conformational changes have been proposed and need to be further evaluated (de Haan et al., 2008; Regan et al., 2008; Regan and Whittaker, 2008; Van Hamme et al., 2007; Van Hamme et al., 2008). Notwithstanding, both S- and M-protein fulfil a crucial role in the internalization process of monocytes/macrophages (Kida et al., 2000).

Replication

CoV genome replication takes place in intracellular double membrane vesicles (DMVs) in the cytoplasm that contain all cellular and viral proteins needed for viral RNA synthesis and shield virus replication against degradation by the cell (Enjuanes et al., 2006; Knoops et al., 2008; Verheije et al., 2010). After genome uncoating, host cell ribosomes can directly translate the positive-stranded RNA to polyprotein replicase precursors which are autoproteolytically cleaved into 16 nonstructural proteins (nsp). These nsp jointly form the membrane-bound replicase-transcription complex (RTC) which is anchored to the DMVs (Figure 1.3 and Figure 1.4 step 4-5). The RTC mediates the synthesis of full-length negative-stranded RNA, which is the template for the synthesis of progeny virus genomes (replication) and the discontinuous synthesis of subgenomic (sg) mRNAs (transcription) (Figure 1.3 and Figure 1.4 step 6) (Gorbalenya et al., 2006; Lai et al., 1982a, b; Siddell, 1995; Spaan et al., 1988). Evidence has been provided that the 5' terminal 55 nucleotides (comprising a short conserved leader sequence 5'CGAAGAGC-3') plus the 3' poly[A] tail are required for the initiation of RNA synthesis (Lai, 1990; Lin et al., 1994). At least one structural protein, N protein, and several nsps (i.e. nsp2, nsp3, nsp9), together with a number of host factors have been shown to interact with these RNA synthesis signals (Almazan et al., 2004; Egloff et al., 2004; Gadlage et al., 2008; Graham et al., 2005; Hurst et al., 2010; Neuman et al., 2008; Schelle et al., 2005; Sims et al., 2000; Sutton et al., 2004; Verheije et al., 2010).

Transcription

CoV transcription is characterized by the generation of 3' coterminal nested sets of negative-stranded subgenomic (sg) mRNAs which are synthesized in different but constant quantities (Figure 1.3) (Siddell et al., 1983; Spaan et al., 1988). These negative mRNAs are translocated to the 5' end of the genome by a 'discontinuous extension mechanism', where they can fuse with the common 5' terminal leader sequence (Enjuanes et al., 2006; Lai et al., 1984; Sawicki and Sawicki, 2005; Spaan et al., 1983). This fusion is guided by the complementarities between the 3' end of this leader sequence and a short characteristic AU-rich sequence

preceding each gene, termed as transcription regulation sequence (TRS) (Bredenbeek et al., 1987; Bredenbeek et al., 1986; Brown et al., 1986; Shieh et al., 1987). Assisted by RNA chaperones, the TRSs act as attenuation signals, inducing template switching and the production of subgenomic minus strands (Enjuanes et al., 2006; Gorbalenya et al., 2006; Schaad and Baric, 1994). These negative-sense RNAs are subsequently used as templates for the synthesis of complementary positive stranded sg mRNAs (Sawicki et al., 2001).

Translation

Typically, CoV can hijack the host translation machinery as their mRNAs are structurally similar to those of their eukaryotic hosts (Enjuanes et al., 2006; Siddell, 1995). This means that, with a few notable exceptions, only the 5'-extreme ORF of each polycistronic sg mRNA is expressed by a cap-dependent ribosomal scanning (Figure 1.3 and Figure 1.4 step 7) (Gorbalenya et al., 2006; Siddell et al., 1983). Nevertheless, some CoV mRNAs contain more than one translational active ORF, which appear to be translated by alternative strategies, including ribosomal frameshifting, leaky scanning and internal entry by ribosomes (Bredenbeek et al., 1990; Brierley et al., 1991; Herold and Siddell, 1993; Liu et al., 1991; Liu and Inglis, 1992a, b; Smith et al., 1990; Thiel and Siddell, 1994).

In FCoV infected cells, N protein production is located in the cytosol, while the membrane-associated structural proteins S, M and E are synthesized on the rough ER (rER) and co-translationally integrated in the ER membrane.

Budding

Newly formed genomes associate with the cytoplasmatic N proteins to create a nucleocapsid structure (Figure 1.4 step 7b). The presence of the E and N proteins and their interaction with the M proteins results in virus budding with the formation of a nucleocapsid enclosed by double-membrane envelope, containing the structural proteins S, M and E (Arndt et al., 2010; de Haan et al., 2000; Lim and Liu, 2001; Narayanan et al., 2000). This virus budding occurs at small, smooth vesicles lying between the rER and the cis side of the Golgi complex, referred to as ER-to-Golgi intermediate compartment (ERGIC) (Figure 1.4 step 8) (Opstelten et al., 1995; Siddell, 1995). Virions are transported from their site of synthesis by vesicular carriers of the exocytotic pathway, to eventually be release by merging of the vesicles with the plasma membrane (Figure 1.4 step 9-10).

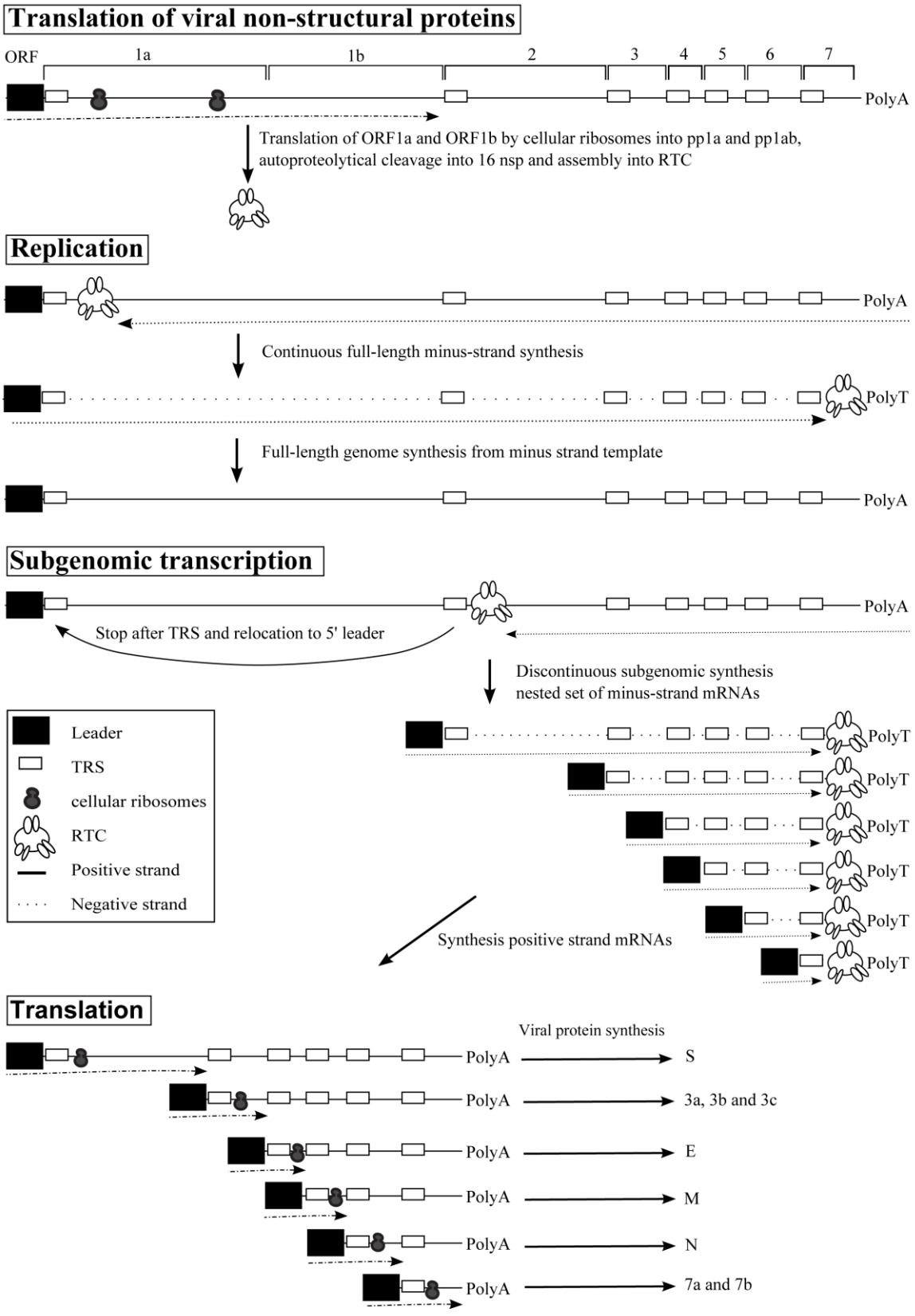


Figure 1.3: Viral replication, subgenomic transcription and translation. ORF: open reading frame; pp: polyprotein; TRS: Transcription-regulation sequence; RTC: replication-transcription complex (Adapted from Vermeulen, 2013)

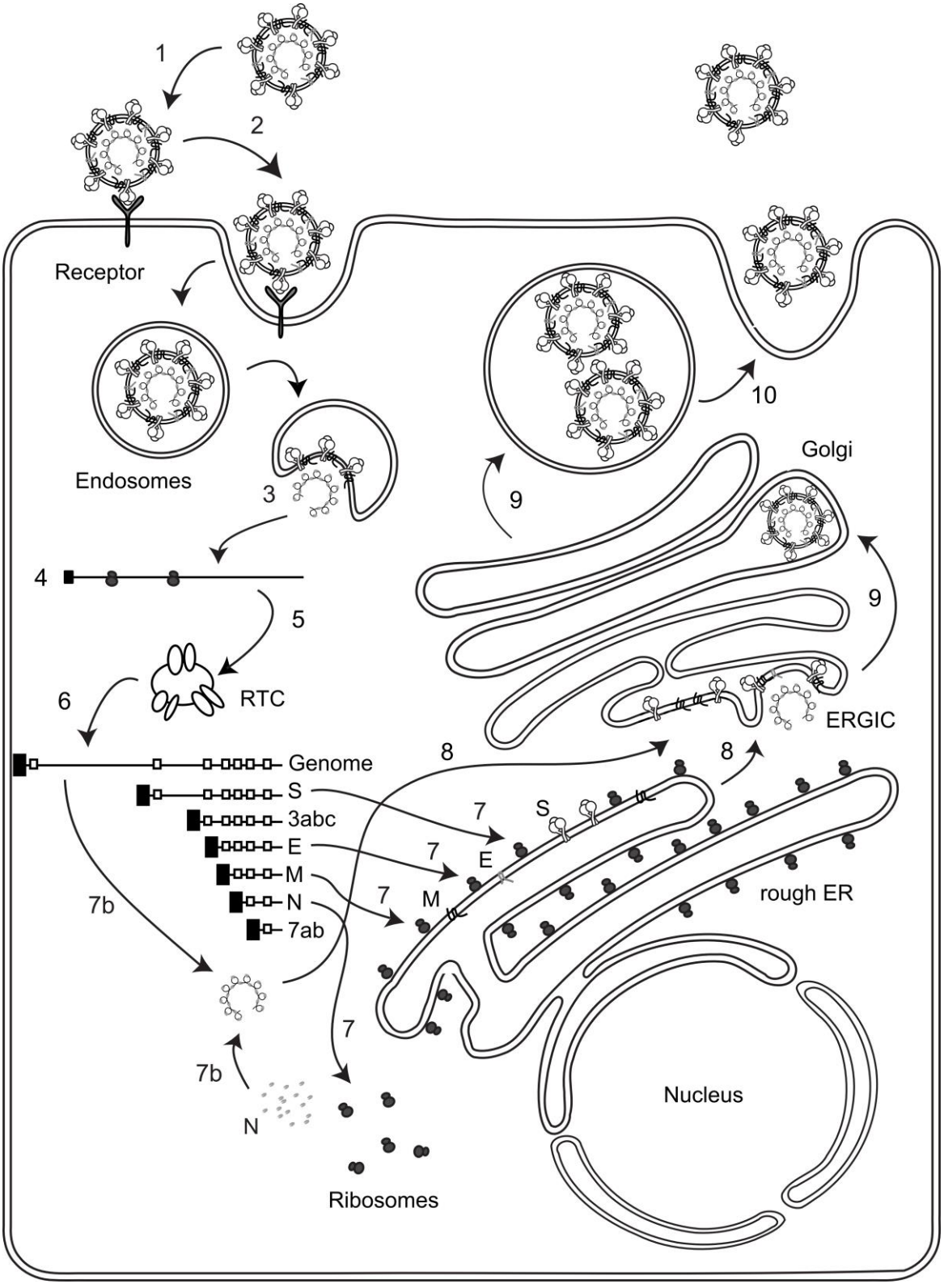


Figure 1.4: Replication cycle of FCoV: 1-2 Binding and internalization. 3 Genome release. 4-5 Translation of the 16 nsp that form the RTC complex. 6 Genome synthesis and mRNA transcription. 7 mRNA translation. 7b Formation of the ribonucleocapsid. 8 Accumulation of the viral proteins in the ERGIC and budding of new virions. 9-10 Transport via the secretory pathway and release of progeny virus (Adapted from Dewerchin, 2008).

1.1.5 Pathogenesis

As the enteric FCoV (**FECV**) is ubiquitous circulating in healthy multicat households, the infection of kittens with FECV is very likely from the age of 5-6 weeks because they are no longer protected by maternal antibodies due to weaning (Addie et al., 2009). Cats infected with FECV will start shedding the virus in their faeces after 1-3 days (Pedersen et al., 2008; Vogel et al., 2010). This virus can then infect other cats by the oral or nasal route and primary starts replicating in the permissive cells in the nasa-oropharyngeal cavity (most likely macrophages or dendritic cells) and enterocytes. From there on, it can spread to the lungs, brain, liver, lymphoid tissue, bone marrow and thymus, but this systemic appearance is far less common than seen with FIPV (Gunn-Moore et al., 1998; Kipar et al., 2010; Meli et al., 2004; Pedersen et al., 1984). The infection with FECV is mostly asymptomatic, or causes at most a mild enteritis with diarrhoea in young kittens (Pedersen et al., 1981a). While some cats will be chronically infected with FECV and turn into a life lasting virus carrier and shedder, most cats can clear the virus after 2-10 months, but will be susceptible for reinfections with the same or a different strain (Foley et al., 1997; Herrewegh et al., 1997; Meli et al., 2004; Pedersen et al., 2008). The different infection courses indicate that the generated immunity is highly variable in strength and duration and lacks memory. Recently, a hypothetical FECV infection model was proposed, characterized by three phases (Vermeulen, 2013). The adapted pathogenesis model was based on an experimental infection study, inoculating three specific pathogen free cats with an infectious field strain FECV-UCD. In a first phase, a low level productive infection of permissive cells (most likely macrophages or DCs) in the naso-oropharyngeal cavity occurs, which allows the virus to spread throughout the host and escape from immune responses. In phase II, the virus passes the gastro-intestinal tract and infects enterocytes, leading to high faecal shedding and activation of the immune system. Phase III is the outcome of virus dissemination by migrating infected cells (DCs) in the naso-oropharyngeal cavity to the gut. The infection study revealed that the second phase can be skipped by the virus.

Although FECV infections may themselves be of low clinical and economical importance, they are the source of the highly virulent and fatal **FIPV**, which likely arises by mutation from FECV within 5-12% of the seropositive cats (Poland et al., 1996; Vennema et al., 1998). Possible responsible mutations are located in the 3c gene and the S gene (Chang et al., 2010; Chang et al., 2012; Pedersen et al., 2012; Rottier et al., 2005; Vennema et al., 1998). The mutation to FIPV is accompanied by a change in virus tropism to the monocytes-macrophage

population, resulting in a far more efficient replication in these cell types and inducing a highly systemic spread of the virus (Dewerchin et al., 2005; Rottier et al., 2005; Stoddart and Scott, 1989). Infected monocytes become activated and typically mount the expression of cell adhesion molecules, like the β_2 integrin macrophage-1 antigen (Mac-1) (Olyslaegers et al., 2013). This mediates the adhesion to endothelial cells, which induces the expression of cytokines, like tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL1β), strengthening the adhesion (Kipar et al., 2005). By production of matrix metalloproteinase B, the vascular basal laminae are destroyed and the monocytes can now migrate to the underlying body cavities, where they accumulate in perivascular granulomas. The subsequent inflammation reactions are associated with the release of chemotactic mediators which on their turn will attract more target monocytes. This results in vasculitis and possible leakage. They are restricted to small and medium-sized veins, mainly in the intestines, lungs, liver, renal cortex and eyes (Kipar et al., 2005).

The ante-mortem early diagnosis of feline infectious peritonitis (FIP) in cats remains a challenge in clinical practice (Diaz and Poma, 2009). Many other diseases can lead to similar clinical symptoms and haematological and biochemical parameters as for FIP (feline leukaemia virus disease (FeLV), feline immunodeficiency virus disease (FIV), neoplasia, some liver and kidney diseases, toxoplasmosis, lysosomal storage disease, trauma, bacterial meningitis,…) (Willoughby et al., n.d.). The early, non-characteristic clinical signs caused by FIPV are depression, chronic fluctuating fever, weight loss and anorexia. Ultimately, FIP results in death of the affected animal as a consequence of the vasculitis, granulomatous lesions and organ failure (Olsen, 1993). The presence of typical viscous yellow effusions in one or more body cavities (like abdomen and thorax) indicates the development of 'wet' FIP. Usually, the wet form of FIP shows a more acute disease pattern and cats will die within weeks or a couple of months (Olsen, 1993). Cats displaying the dry form of FIP develop no exudates and larger granulomas (Pedersen, 1987). This type of disease appears to be more commonly involved in damaging nervous tissues, often characterized by neurological clinical signs (Rand et al., 1994). Cats with dry FIP can survive over a period of several month, indicating a slower progression of the disease.

1.1.6 Induction and evasion of the host immunity by feline coronaviruses

Innate immunity

As coronavirus replication occurs in the cytoplasm, these viruses can immediately be recognized by the host cell, leading to the instant activation of a non-specific innate immune

response. The recognition capacity by the innate immunity relies on a limited number of 'pattern recognition receptors' (PRRs), which allows the discrimination between self and non-self infectious molecules. The major outcome of the signalling cascade induced by these PRRs stimulation, is the production of inflammatory cytokines (TNF-α, interferons (IFNs), interleukins (ILs), chemokines). These cytokines promote resistance of cells against viral replication and recruit plasma proteins (e.g. complement proteins) and immune cells to sites of infection (Blach-Olszewska, 2005; Janeway and Medzhitov, 2002). The attracted immune cells can directly destroy pathogens and virus-infected cells (neutrophils and natural killer (NK) cells) or will maturate and eventually direct the adaptive immunity, through a process known as antigen presentation (tissue macrophages and dentritic cells (DCs)) (Janeway and Medzhitov, 2002). Type I interferons (IFNα/β) are one of the most important virally induced cytokines produced by the innate immunity. They are known to elicit distinct antiviral, immunomodulatory and antiproliferative effects in infected and neighbouring uninfected cells (O'Shea et al., 2004; Platanias, 2005), which will be discussed in detail in part 1.2 . The innate immunity inhibits efficient virus replication, allowing the host to generate the slower adaptive immune response, which might protect against subsequent challenges (Tosi, 2005). The speed and efficiency by which a virus can antagonize the innate immunity may be critical determinants in its host range and pathogenicity (Goodbourn et al., 2000). At present, there is still limited knowledge about the effectiveness of the innate immunity during FCoV infections and the possible evasion strategies FIPV is using.

Adaptive immunity

The adaptive immune response is a compulsory second branch of the immune defence against viral intruders, especially when the innate immunity has failed to overcome the infection. It becomes effective after 4-5 days and allows for a stronger antigen-specific immune response which is tailored to specific pathogens resulting in immunological memory (Chaplin, 2003).

The adaptive humoral immunity is characterized by the production of antibodies which recognize viral particles and viral proteins that are expressed on the cell membrane of the infected cells. This directs cell lysis via binding and activation of the complement system ('antibody-dependent complement mediated lysis' (ADCML)) (Sissons and Oldstone, 1980) or via activated NK cells or phagocytic cells ('antibody-dependent cell-mediated cytotoxicity' ADCC) (Koren, 1983). FIPV infection induces the humoral immune response as high amounts of virus-neutralizing antibodies as well as virus-specific antibody-producing cells are detected in blood and lesions (de Groot-Mijnes et al., 2005; Kipar et al., 1998;

Takano et al., 2009). Nevertheless, the humoral immune system fails to control or clear the infection and may even contribute to the pathogenesis of FIP (Paltrinieri et al., 1998). This can be attributed to following immune evasion strategies of FIPV:

i. The binding of antibodies on cell free FIPV may facilitate virus entry into monocytes/macrophages via Fc receptor mediated internalization (Hohdatsu et al., 1991; Olsen et al., 1992; Takano et al., 2008). As FIPV is able to replicate in monocytes/macrophages upon escape from the phagocytic compartment, its infection and spread may be enhanced instead of inhibited. This mechanism is called 'antibody-dependent enhancement of infectivity' (ADEI) and has up to now been reported for *in vitro* and experimental infections only (Addie et al., 1995; Pedersen and Black, 1983; Takano et al., 2008; Vennema et al., 1990).
ii. FIPV infected cells may remain invisible for the humoral immune response by two strategies. On the one hand, FIPV avoids the presence of viral antigens on the plasma membrane of 50% of the infected cells by a mechanism called intracellular retention (Cornelissen et al., 2007). On the other hand, the remaining 50% of infected cells will rapidly internalize their cell-surface expressed viral antigens upon binding of specific antibodies (Dewerchin et al., 2006). These immune evasion strategies protect the cell against ADCML and ADCC.
iii. It has been shown that ADCML is inhibited even if antibodies get the chance to recognize the cell by artificially blocking the internalisation process. As the accessory proteins are not important in this process, the structural or non-structural proteins encoded by ORF1ab are likely responsible (Cornelissen et al., 2009).

In contrast to the humoral response, it is generally believed that a strong **cell-mediated adaptive immune response** can protect cats from developing FIP (Pedersen and Black, 1983). The presentation of viral peptides on class I major histocompatibility complex (MHC-I) molecules enables cluster of differentiation 8 (CD8)+ cytotoxic T-lymphocytes to recognize and destroy the infected cell. The more efficient this T-cell response, the better the prognosis of the infection (de Groot-Mijnes et al., 2005). Nevertheless, FIPV is also counteracting the cell-mediated immunity. In cases where FIP does manifest, an early depletion of NK cells, T-, B- and regulatory T-cells is observed and correlates with enhanced viral replication (Kipar et al., 2001; Paltrinieri et al., 2003; Vermeulen, 2013; Vermeulen et al., 2013). Further research showed that apoptosis induced by a soluble molecule (possibly TNF-α or FCoV encoded accessory protein 7b) was probably responsible for the depletion

(Haagmans et al., 1996; Takano et al., 2007). Next to the T-cell depletion, a downregulation of IFN-γ and lower levels of IL12 in FIP cats could correlate with an impaired cell-mediated immunity (Gelain et al., 2006; Gunn-Moore et al., 1998; Kipar et al., 2006; Kiss et al., 2004; Vermeulen, 2013). Last, FIP LN-derived NK cells showed a higher activated phenotype but had significantly less cytotoxic capacity (Vermeulen et al., 2013), a strategy that has also been evolved by some other viruses (e.g. HIV and HCV) (Meier et al., 2005).

It appears that FIP is an outcome of a complicated balance between host immune responses and FIPV evasion strategies. To clarify the FCoV pathogenesis, many questions still have to be solved concerning the interactions of the virus with both the innate and adaptive immune system.

1.1.7 Prevention and treatment

Prevention

At present, the only commercial available vaccine against FIP is a temperature-sensitive mutant of type II FIPV DF-2 (Primucell FIP from Pfizer Animal Health). This mutant is only capable of replicating at 31°C in the upper respiratory tract but fails in maturation and assembly at 39°C, which is the local temperature on systemic places in the cat (Christianson et al., 1989; Gerber et al., 1990a; Gerber et al., 1990b). The vaccine needs to be administered two times intranasally, spread over 3 weeks. The vaccine stimulates the cellular immunity and mucosal immunity against FCoV by a local IgA response of the mucosal B lymphocytes which protects against viral invasion and subsequent FIPV-induced immune-mediated pathology. However, the results of different experimental and field studies are inconsistent with 0 to 75% prevention of disease (Fehr et al., 1995; Fehr et al., 1997; Hoskins et al., 1995; Mcardle et al., 1995; Scott et al., 1995). This can mostly be ascribed to the fact that the vaccine is not functional in FCoV positive cats and that vaccination can only be started from 16 weeks of age. As most kittens are infected with FCoV before the age of 16 weeks, the vaccine is often not useful. Moreover, vaccination should be repeated frequently (every 6-9 months).

In 2004 Haijema et al. published promising results when vaccinating with attenuated type II FIPV strains deleted in their accessory proteins 3abc and 7ab (Haijema et al., 2004). Vaccinated cats were protected against lethal homologous challenge. However, up till now, these hopeful vaccines are still not commercially available, additionally no studies were reported concerning their protection capacity against the more prevalent type I FCoVs.

As FIPV may arise from FECV, the best way to prevent FIP development is eradicating FECV from multicat households by separating seropositive cats from seronegative cats and maintaining a good hygiene. In addition, reducing the infection pressure by avoiding large numbers of cats and reducing stress, can minimize the spread of FCoV (Addie et al., 2009; Addie and Jarrett, 1990).

Treatment

When a cat is diagnosed with FIP, no effective treatment can save this cat from death. Still, at early stages, some immunosuppressive (like glucocorticoids) and anti-inflammatory drugs can slow down the progression of the disease. Both ozagrel hydrochloride (thromboxane synthetase inhibitor) and ribavirin (antiviral drug) were promising therapeutic components but could not be used *in vivo* due to negative side effects or even severe toxicity, respectively (Watari et al., 1998; Weiss et al., 1993). Also interferon-ω has been tried but inconsistent results were reported (Ishida et al., 2004; Ritz et al., 2007). The treatment of FIP with interferon-α in combination with Propionibacterium acnes prolonged the mean survival time only for a couple of days (Weiss et al., 1990). The real efficacy of all these drugs is difficult to evaluate, as most studies did not enclose appropriate controls or had no confirmation of FIP (Hartmann and Ritz, 2008).

1.2 Antiviral activity of interferon against positive single stranded RNA viruses

1.2.1 Introduction

Interferons (IFNs) were discovered more than 50 years ago (Isaacs and Lindenmann, 1957) and are now known to be the key players in establishing an early powerful host defence. By controlling the expression of more than 300 genes, they possess a wide range of biological activities that are essential for the host to eradicate an invading pathogen, particularly viruses. Stimulating the 'antiviral state' of the target cell is the by far most studied effect of interferons, but also cell growth regulation, differentiation and apoptosis, as well as exerting immunomodulatory effects (e.g. enhancing Class I and II MHC molecules expression) and stimulating the adaptive immune response (e.g. increasing T-lymphocyte activity) can be added to their action list (Goodbourn et al., 2000; Meyer, 2009; Randall and Goodbourn, 2008; Samuel, 2001; Yoneyama and Fujita, 2010).

Three distinct types can be distinguished among the IFNs, with each of them having their own characteristic receptor and signalling pathway (Meyer, 2009; Randall and Goodbourn, 2008). Type I IFNs consist mainly of IFN-α and IFN-β which play a primary role in the innate immunity against viruses. They can be produced by most types of virus infected cells and are predominantly induced by plasmacytoid dendritic cells (pDCs). Beside these, also other less functionally defined variants such as IFN-ω, -ε, -τ, -δ and -κ complete this multigene family. Type II IFN, also known as 'immune IFN', has a single member, IFN-γ, and is induced by mitogenic or antigenic stimuli (i.e. factors that activate lymphocytes). IFN-γ is synthesized by specific immune cells including NK cells, CD4 Th1 cells and CD8 cytotoxic suppressor cells, highlighting its role in the adaptive cellular immune response (Goodbourn et al., 2000; Meyer, 2009; Randall and Goodbourn, 2008; Samuel, 2001). Type III IFN- λ1, - λ2 and –λ3 (or IL-28A, IL-28B and IL-29) are also induced in direct response to viral infection and appear to use the same pathway as the IFN-α/β genes to sense viral infections (Meyer, 2009; Onoguchi et al., 2007). This novel class of cytokines seems to be primary important at epithelial surfaces (Ank and Paludan, 2009).

In the next paragraphs (see also Figure 1.5), focus will be put on IFN-α/β production by cells infected with positive single stranded RNA viruses, how IFN-α/β induce the transcription of their target genes and how these target genes exert their antiviral effects.

1.2.2 Induction of type I IFN

In normal cells, the expression of IFN genes is inhibited by the binding of a repressor protein on the promoter region. Upon microbial invasion of the host cell, non-self nucleic acids, lipopolysaccharide (LPS) or bacterial components (known as pathogen-associated molecular patterns or 'PAMPs') can be recognized by germline-encoded 'pattern recognition receptors' (PRRs). These PRR-PAMP interactions induce the expression of i.e. type I IFN by either preventing synthesis of the repressor protein or increasing the levels of activator proteins. Among viruses, the RNA viruses generally are the most potent IFN inducers (O'Neill and Bowie, 2010; Takeuchi and Akira, 2010; Wilkins and Gale, 2010; Yoneyama and Fujita, 2010). Sensing of viral nucleic acids can be mediated by several PRR families which are the transmembrane proteins called 'Toll-like receptors' (TLRs), located in cell and endolysosomal membranes and 'stimulator of interferon genes' (STING), associated with ER membranes and the cytoplasmic proteins being the 'Retinoic acid inducible gene (RIG)-I like receptors' (RLRs) (Figure 1.5) (O'Neill and Bowie, 2010; Saito and Gale, 2008; Takeuchi and Akira, 2010; Yoneyama and Fujita, 2010).

Toll-like receptors (TLRs)

A set of TLRs (TLR3, 7, 8, 9) are specialized in the recognition of viral nucleic acids and are predominantly expressed in the endosomal compartments of immune cells. The uptake of viral particles by endocytosis followed by their endosomal degradation is required to expose the viral RNA to the TLRs (Garcia-Sastre and Biron, 2006; Takeuchi and Akira, 2010; Yoneyama and Fujita, 2010). TLR3 typically recognizes dsRNA, while TLR7 and 8 are known to be sensors for ssRNA. TLR9 is the receptor needed for DNA recognition. The downstream signalling cascade is initiated by binding of the Toll/interleukin-1 receptor (TIR) domain on the TLRs with specific adapter proteins being 'TIR domain-containing adapter inducing IFN-β' (TRIF) for TLR3 and 'myeloid differentiation primary response protein' (MyD88) for TLR7, 8 and 9. Association of the TLRs with their adapter proteins recruits cellular kinases which eventually lead to the regulation of transcription factors which bind to specific motifs in the promoter region of IFN-α/β and inflammatory cytokines (Kawai and Akira, 2006; Takeuchi and Akira, 2010).

Stimulator of interferon genes (STING)

This recently identified molecule is indispensable for sensing cytoplasmic DNA species of several DNA viruses, including HSV-1 and adenoviruses, and activates the cellular defence

mechanisms and expression of IFNs (Abe et al., 2013; Ishikawa et al., 2009; Lam et al. 2013). As STING itself has a very low affinity for DNA, it requires another PRR for DNA sensing, being cyclic GMP-AMP synthase (cGAS). cGAS catalyses the production of cyclic GMP-AMP (cGAMP), which binds to STING. This results in the activation of the IRF-3 (interferon regulatory factor-3) antiviral pathway, inhibiting infection of several viruses, independently of canonical IFN signalling (Kato et al., 2013; Schoggins et al., 2014; Sun et al., 2013; Wu et al., 2013).

RIG-I like receptors (RLRs)

The three RLRs, retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) are responsible for viral RNA detection in the cytoplasm of infected cells (Yoneyama and Fujita, 2010). RIG-I recognizes relatively short dsRNA (up to 1 kb) while MDA5 detects long dsRNA (more than 2 kb). Both possess the ability to induce an antiviral responses (regulated by different transcription factors) via the binding of their 'Caspase Activation and Recruitment Domains' (CARD)-domain with the mitochondrial antiviral signalling protein (MAVS) (Seth et al., 2005; Wilkins and Gale, 2010). LGP2, the remaining RLR, lacks the ability to induce signalling alone (due to the absence of CARD domains), but has been found to be necessary for effective RIG-I and MDA5-mediated antiviral responses (Satoh et al., 2010). Type I IFN expression renders also a positive feedback regulation on RLRs expression (Takeuchi and Akira, 2010; Yoneyama and Fujita, 2010)

Type I IFN expression

There are three types of transcription factors (TFs) that are activated by all previous described PRR-PAMP interactions and which are involved in IFN-α/β expression: IFN regulatory factors (IRFs), nuclear factor kappa B (NF-κB), and activation protein-1 (AP-1) (Figure 1.5). The IFN-β promoter contains motifs for all three TFs, while the promoter of IFN-α genes only contains an IRF binding motif. Among the ten-member IRF family members, IRF-3 and IRF-7 were demonstrated to have an essential role in the activation of IFN-α/β. Their activation is characterized by phosphorylation of C-terminal serine residues, causing them to form a homodimer and unveiling a nuclear-localisation signal (NLS). In the nucleus, they can interact with a transcriptional co-activator, p300/CREB-binding protein (CBP) which specifically recognizes and activates the promoter of type I IFN (Randall and Goodbourn, 2008; Samuel, 2001; Yoneyama and Fujita, 2010). NF-κB is a homo- or heterodimer composed of Rel-like domain-containing proteins with the p65(RelA)-p50 complex as most

abundant prototype. It is retained in the cytoplasm of unstimulated cells in an inactive state by association with its inhibitor IκB. PPR-PAMP interactions induce the phosphorylation of IκB by IκB kinase (IKK). This subsequently initiates the ubiquitination of IκB, which marks this protein for proteolytic degradation by the 26S proteasome. By doing so, the NLS of the p65 subunit becomes accessible and NF-κB is recognized for nuclear translocation (Hayden and Ghosh, 2004; Kawai and Akira, 2006; Randall and Goodbourn, 2008; Wullaert et al., 2006; Yoneyama and Fujita, 2010). AP-1 is a dimeric leucine zipper protein composed of proteins belonging to the c-Jun, c-Fos, ATF (activating transcription factor), JDP (Jun dimerization protein), Maf (musculo-aponeurotic fibrosarcoma) and Nrl (neural retina leucine zipper) subfamilies. AP-1 activation by PRRs is regulated by MAP kinases such as c-jun N terminal kinase (JNK), p38 and extracellular signal-regulated kinase (ERK) (Kawai and Akira, 2006; Yoneyama and Fujita, 2010).

1.2.3 Type I IFN signal transduction

Produced IFN-α/β is secreted by the infected cell and alerts the surrounding cells (paracrine) and its own producer cell (autocrine) via a cognate receptor complex by activation of a signalling cascade mainly regulated by JAK/STAT (Janus Kinase/Signal Transducer Activator of Transcription) pathway proteins (Figure 1.5) (Randall and Goodbourn, 2008; Takeuchi and Akira, 2010; Wilkins and Gale, 2010; Yoneyama and Fujita, 2010). The type I IFN common receptor is composed of two membrane spanning proteins IFNAR1 and IFNAR2, which form a ternary complex upon binding with IFN-α/β. This oligomerization induces the cross-phosphorylation of two Janus-family tyrosine kinases (JAKs); TYK2 and JAK1, associated with the cytoplasmic tail of IFNAR1 and IFNAR2 respectively. Subsequently, the activated JAKs phosphorylate the receptors on their tyrosine residues, creating a strong docking site for STAT1 and STAT2, which are on their turn phosphorylated by the JAKs. The phosphorylated stable STAT1/STAT2 heterodimer then dissociates from the receptor and binds to IRF-9/p48, a member of the Interferon Regulatory Factor-9 family, constituting a heterotrimeric complex called ISGF3 (Interferon-Stimulated Transcription Factor-3) (Meyer, 2009; Samuel, 2001; Yoneyama and Fujita, 2010). In this complex, a novel NLS is created and the dominant constitutive nuclear export signal of STAT2 is inactivated, leading to the translocation of ISGF3 to the nucleus (Randall and Goodbourn, 2008). In the nucleus, it binds to consensus *cis*-acting DNA sequences, designated ISRE (IFN-stimulated response elements), present in the promoters of hundreds of interferon-stimulated genes (ISG), and enhances their transcription (Schoggins et al. 2011, 2014; Liu et al., 2012). Most

ISGs confer robust antiviral activities in the cell by i.e. directly inhibiting viral infection or triggering apoptosis (Samuel, 2001; Takeuchi and Akira, 2010; Wilkins and Gale, 2010; Yoneyama and Fujita, 2010).

Virus induction of IFN-α/β and its antiviral actions can be extremely cell type dependent, as demonstrated for MHV (Rose and Weiss, 2009). The cross talk between separate signalling pathways activated by IFN and other cytokines, together with the orchestrated activities of several cell types will eventually determine the strength of the innate immune response to viral infection (Randall and Goodbourn, 2008; Rose and Weiss, 2009; Samuel, 2001; Stetson and Medzhitov, 2006; Zurney et al., 2007).

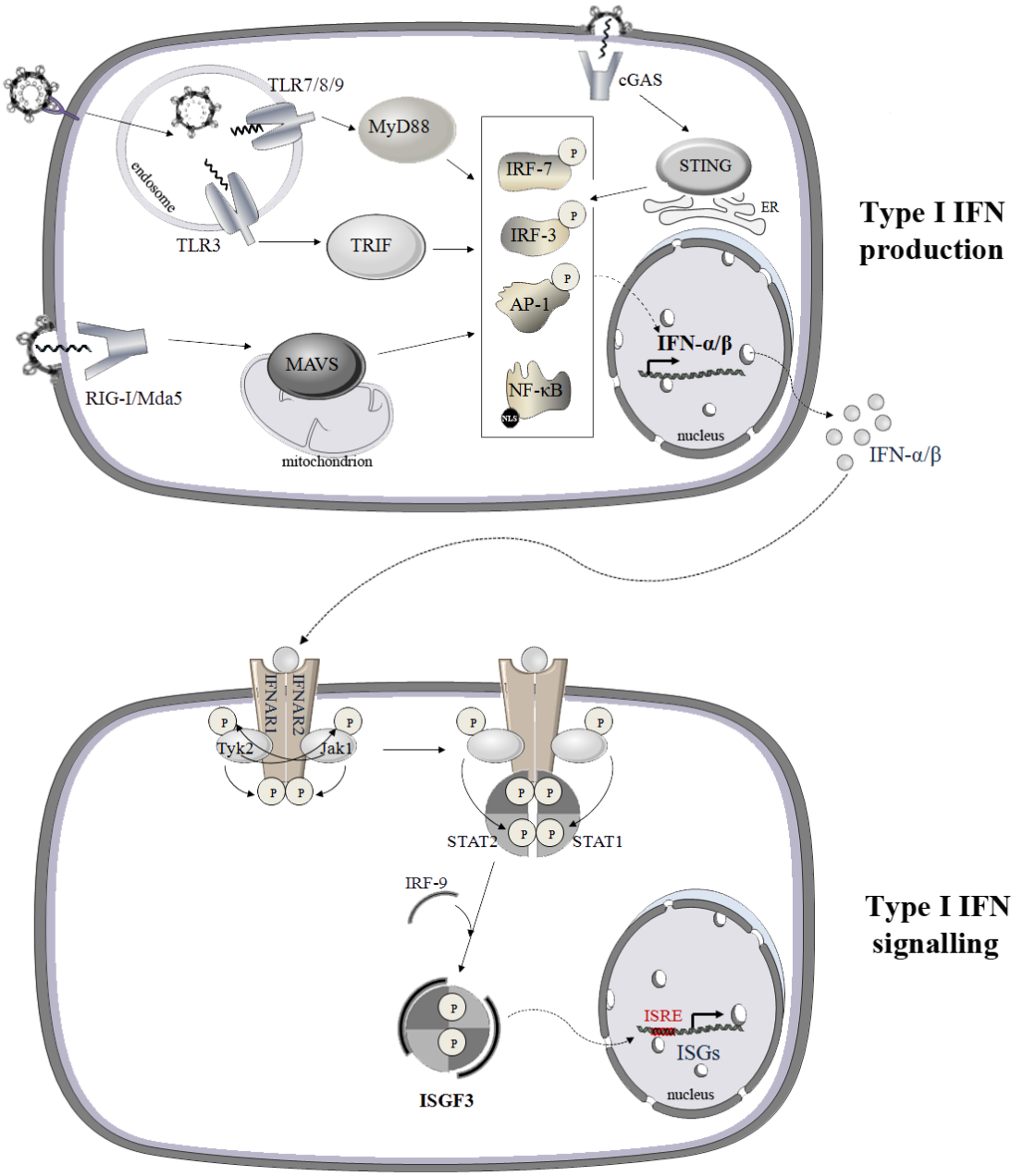


Figure 1.5: Schematic representation of type I IFN production (above) and type I IFN JAK/STAT signalling pathway (below). Pattern recognition receptors (PPRs), like toll-like receptors (TLR), retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5)or cyclic GMP-AMP synthase (cGAS**)** recognize viral nucleic acids. This induces a signalling cascade through different adaptor proteins (e.g. TRIF, MyD88, MAVS and STING) which subsequently activates several transcription factors, including IFN regulatory factors (IRFs), nuclear factor kappa B (NF-κB) and activation protein-1 (AP-1), which will lead to the activation of the type I IFN promoter. IFN-α/β is released by the infected cell and binds to the IFN receptor of surrounding and its own producer cell. A chain of phosphorylations, regulated by JAK/STAT pathway proteins, results in the nuclear translocation of a stable complex (ISGF3) that stimulates the expression of numerous interferon-stimulated genes (ISGs) through the recognition of IFN-stimulated response elements (ISRE) in its promoter.

1.2.4 Antiviral defences induced by IFN-α/β

The antiviral actions of type I IFNs have been studied extensively and the best-characterized IFN-induced enzymes are dsRNA dependent protein kinase (PKR) and 2'-5' oligoadenylate synthetase (i.e. OAS). Their synthesis results in the direct (i.e. PKR) or indirect (OAS) inhibition of protein synthesis. Next to these, also other factors are implicated in the antiviral response, e.g. protein Mx GTPases and adenosine deaminases. All these antivirals collaborate to disrupt multiple stages of the virus lifecycle, minimizing the possibility that a single virus evasion protein could thwart the whole antiviral state (Liu et al., 2012).

Beside the broad range of type I IFN-stimulated gene (ISGs) products that target positive stranded RNA viruses (which are represented in Figure 1.6 and described below), it is worthwhile mentioning some ISGs, including MCOLN2, MAP3K5, INDO and FAM46C, have recently been identified as enhancers of virus infectivity. This underlines the high complexity of the IFN response (Schoggins et al., 2011, 2014).

PKR (IFN-induced dsRNA dependent protein kinase)

PKR is a serine/threonine kinase that is associated with ribosomes in a monomeric inactive state in non-stressed cells (Zhu et al., 1997). It consists of two well characterized domains; an N-terminal regulatory domain that contains two dsRNA binding motifs an a C-terminal catalytic kinase domain (McCormack et al., 1994; Meurs et al., 1990; Proud, 1995). PKR is predominantly activated upon binding to dsRNA with sizes of at least 30nt, sourced either viral, cellular or synthetic (Clemens and Elia, 1997; Garcia et al., 2007). This induces a conformational change that leads to unmasking of the kinase domain, followed by a homodimerization and autophosphorylation. This is typically inhibited by high dsRNA concentrations due to saturation of the dsRNA binding sites (Clemens et al., 1993; Goodbourn et al., 2000; Samuel, 2001). Activated PKR can induce the phosphorylation of at least six proteins, among which the α subunit of eukaryotic translation initiation factor 2 (eIF2α), leading to inhibition of protein synthesis, is best characterized. Moreover, it plays an important role in the signal transduction and transcriptional control through the NF-κB pathway as well as in the control of cell growth and apoptosis, as discussed below (Garcia et al., 2007; Goodbourn et al., 2000; Samuel, 2001). Although there is abundant evidence that PKR plays a major role in regulating virus infection, PKR is not sufficient to mediate the full antiviral response (Goodbourn et al., 2000; Yang et al., 1995).

As mentioned above, activated PKR is one of the main regulators of the host translational mechanism by phosphorylation of eIF2α protein synthesis factor at serine 51. In eukaryotic

cells, protein synthesis is a complex process regulated by different translation initiation factors. eIF2α is one of the key players and is a GTP-binding protein that mediates binding of the initiator Met-tRNA to the 40S ribosome subunit. Other initiation factors will bind and transport the mRNA to this complex. The initiator complex starts the scanning of the mRNA until the Met-tRNA interacts with the initiator AUG codon. Subsequently, GTP is hydrolyzed from the eIF2α, initiation factors are released, and the 60S ribosomal subunit joins the complex whereupon elongation is started. The released eIF2α-GDP can be used for another round of translation initiation by exchanging the GDP for GTP, mediated by the guanine-exchange-factor eIF2B. Phosphorylated eIF2α has an increased affinity for eIF2B, leading to its irreversible binding to eIF2B, whereby preventing the recycling of eIF2α-GTP (Clemens and Elia, 1997; Garcia et al., 2007; Goodbourn et al., 2000; Ramaiah et al., 1994; Samuel, 2001). Since eIF2B is present in limiting amounts with respect to eIF2α, the cellular translational machinery is halted by small increases in eIF2α phosphorylation and viral protein synthesis and replication are restricted within the infected cells (Garcia et al., 2007; Goodbourn et al., 2000; Proud, 2005). Phosphorylation of eIF2α represents one of the potent antiviral consequences of PKR activation, targeting a wide spectrum of DNA and RNA viruses (Garcia et al., 2007). Interestingly, while inhibiting translation of most mRNAs, translation of a few selected transcripts (mostly functioning in the unfolded protein response UPR) is enhanced upon eIF2α phosphorylation. These proteins may indirectly contribute to the antiviral response (Brush et al., 2003; Harding et al., 2000; Stetson and Medzhitov, 2006). PKR also aids in virus clearance by mediating signal transduction and regulating transcription of for instance IFN-β, IRF-1 and NF-κB (Kumar et al., 1994; Kumar et al., 1997; Zinn et al., 1988). Evidence is consistent with a role for PKR in the activation of IKK, but the precise mechanism remains to be clarified (Gil et al., 2004; Gil et al., 2001; Zamanian-Daryoush et al., 2000). Activated IKK liberates NF-κB from its inhibitor IκB, which then translocates to the nucleus, inducing the expression of inflammatory cytokines and enchancing MHC Class I presentation. Furthermore, PKR can provoke apoptosis to destroy the cell before the virus can fully replicate and assemble (Garcia et al., 2007; Goodbourn et al., 2000; Kaufman, 1999; Lee et al., 1997; Zhang and Samuel, 2007).

OAS (2'-5'-oligoadenylate synthetase)

As for PKR, infection and subsequent IFN signalling increases the basal levels of OAS, which is further activated by binding to viral dsRNA (Castelli et al., 1998b; Randall and Goodbourn, 2008). The dsRNA binding domain of OAS has no structural homology with that

of PKR, but like PKR, OAS does possess separate subdomain regions responsible for RNA-binding and catalytic activity (Ghosh et al., 1991; McCormack et al., 1994). Activation allows OAS to convert ATP into short oligoadenylates, containing unusual 2'-5' phosphodiester bonds, collectively referred to as 2-5A (Kerr and Brown, 1978). 2-5A oligonucleotides bind strongly to pre-existing endoribonuclease L (RNaseL) and induce the stable formation of homodimers which acquire full catalytic activity (Dong and Silverman, 1995; Dong et al., 1994; Verheijen et al., 1999). Activated RNaseL instigates the cleavage of viral and cellular mRNA, as well as 28S ribosomal RNA, leading to the inhibition of viral protein synthesis (Dyer and Rosenberg, 2006; Iordanov et al., 2000; Samuel, 2001; Silverman, 2007). This mechanism operates using a positive feedback loop, whereby increasing amounts of viral dsRNA consequently activate additional RNaseL. The OAS/RNaseL system can eventually activate apoptotic pathways to avoid widespread viral dissemination (Castelli et al., 1998a; Castelli et al., 1998b; Diaz-Guerra et al., 1997; Zhou et al., 1997). In any case, the overall activation status of this system is depending on the type of IFN, the type of cell, the growth state of the cell and the viral invader (Samuel, 2001).

ADAR

Adenosine deaminase acting on RNA (ADAR) are again a family of dsRNA-binding proteins and they alter viral and cellular genetic information through conversion of adenosines (A) to inosines (I) in dsRNA (Bass and Weintraub, 1988; Polson and Bass, 1994; Wagner et al., 1989). The details of the molecular mechanisms by which ADARs modulate virus-host interactions largely remain unresolved, but a lot of RNA and even DNA viruses are implicated as editing targets (Samuel, 2011). Viral replication can be disrupted because A-to-I editing can (i) cause the dsRNA to unwind as I:U mismatches are less stable than A:U base pairs or (ii) activate an inosine specific ribonuclease which will efficiently cleave the inosine-containing RNA (Scadden and Smith, 1997). Additionally, I is read as G instead of A by ribosomes during translation and by viral RNA-dependent RNA polymerases during RNA replication, leading to altered protein translation/activity and viral genome mutations, respectively (Samuel, 2012). On the other hand, it has been reported that this RNA editing enzyme is also stimulating infection of certain viruses, like HIV-1, CHIKV and measles virus (Doria et al., 2009; Schoggins et al., 2011; Toth et al., 2009).

APOBEC

The conserved APOBEC ("apolipoprotein B mRNA editing enzyme-catalytic polypeptide") family are members of another class of RNA-editing enzymes. Their mechanism of action consists both of viral genome mutation by cytidine (C) to uridine (U) editing (=cytidine deamination) as well as of disruption of viral replication by inhibition of reverse transcription (Dyer and Rosenberg, 2006). The best characterized protein is APOBEC3G, for which it has been shown that it can incorporate into retroviral as well as coronaviral virions, by association with structural viral proteins, leading to restricted infectivity and spread (Ao et al., 2008; Randall and Goodbourn, 2008; Wang and Wang, 2009).

Protein Mx

The myxovirus resistance (Mx) proteins are members of the dynamin superfamily of highly conserved, large guanosine triphosphatases (GTPases) (Haller et al., 1998; Kochs et al., 1998; Landis et al., 1998; Staeheli et al., 1993). Intensive research has revealed that they possess antiviral properties against a wide range of viruses from different taxonomic groups, with distinct structural architecture (e.g. negative and positive single stranded RNA viruses, double-stranded RNA viruses and DNA viruses) and diverse replication locations (e.g. nucleus versus cytoplasmic) (Haller et al., 2007; Kochs et al., 1998; Landis et al., 1998; Larsen et al., 2004; Netherton et al., 2009). Several reports support a direct interaction of Mx proteins with viral targets (e.g. nucleocapsid or polymerase proteins), thereby modifying their localisation or activity, resulting in a restricted virus replication or assembly (Kochs and Haller, 1999a, b; Landis et al., 1998; Lee and Vidal, 2002; Pavlovic et al., 1993; Stranden et al., 1993; Weber et al., 2000). A functional N-terminal GTP binding domain and intact C-terminal part of the Mx proteins seem to be required for antiviral function (Haller et al., 1998; Pitossi et al., 1993).

ISG15, ISG54 and ISG56

Following IFN stimulation, there are also a subset of genes upregulated whose biological functions are still not clearly defined and which were named according to the molecular mass of the protein (Randall and Goodbourn, 2008).

ISG15 is the earliest and most strongly induced ISG and represents a 15 kDa ubiquitin-like polypeptide (Giannakopoulos et al., 2005; Haas et al., 1987; Harty et al., 2009; Kerscher et al., 2006; Narasimhan et al., 2005). The two tandem ubiquitin-like domains and C-terminal LRLRGG sequence declare its ability to conjugate to lysine residues on cellular and viral proteins (Chua et al., 2009; Giannakopoulos et al., 2005; Narasimhan et al., 2005; Zhao et al.,

2005). Similar to ubiquitin, the ATP-dependent conjugation of ISG15 (=ISGylation) requires the cooperative activities of three classes of enzymes, being E1 (UbE1L), E2 (UbcH8) and E3 (e.g. Herc5) (Dastur et al., 2006; Harty et al., 2009; Zhao et al., 2004). By targeting key components of many fundamental pathways, ISG15 modification has significant effects on cap-dependent translation, glycolysis and cell motility (Giannakopoulos et al., 2005; Okumura et al., 2007) as well as on the antiviral IFN response (including JAK/STAT, NF-κB, IRF-3 and RIG-I signalling pathways (Chua et al., 2009; Kim et al., 2008; Lenschow et al., 2007; Lu et al., 2006; Malakhov et al., 2003; Takeuchi et al., 2006; Zhao et al., 2005)) against both DNA and RNA viruses, like influenza, herpes, sindbis, vaccinia virus and HIV-1 (Guerra et al., 2008; Harty et al., 2009; Hsiang et al., 2009; Lenschow, 2010; Okumura et al., 2006). To date, it is not clear how ISGylation modulates the protein activities, but it does not appear that it targets proteins for proteasomal degradation (Giannakopoulos et al., 2005; Harty et al., 2009; Lu et al., 2006; Zhao et al., 2004). Next to this conjugation dependent role of ISG15, an immunoregulatory role on Chikungunya virus (CHIKV) infection has been described which does not require ISGylation (Werneke et al., 2011). Furthermore it could also act as an extra-cellular cytokine for which its activity *in vivo* needs to be ascertained (D'Cunha et al., 1996; Lenschow, 2010; Owhashi et al., 2003; Padovan et al., 2002).

ISG54 and ISG56 are related interferon-induced proteins with tetratricopeptide repeats (IFIT) that play important roles in blocking viral replication and regulating cellular functions, like apoptosis (Stawowczyk et al., 2011). The tetratricopeptide repeat is a structural motif that facilitates protein-protein interactions and often assembly of multiprotein complexes (Blatch and Lassle, 1999). Different antiviral mechanisms have been ascribed to them. Early studies reported that they inhibited translation by binding the translation initiation factor eIF3, inhibiting its ability to stabilize the eIF2-GFP-tRNAiMet complex and to form the 48S pre-initiation complex (Terenzi et al., 2006). Later, it was demonstrated that the lack of 2'-O-methylation of the 5'cap of particular viral mRNAs is stimulating the antiviral capacity of IFIT members and induces translation suppression (Daffis et al., 2010). Recently, it was reported that IFIT members recognize and bind viral mRNA carrying a triphosphate group on their 5' terminus, thereby antagonizing viral replication (Pichlmair et al., 2011). The crystal structure of ISG56 family members confirmed the ability of these proteins to bind specifically to some RNAs and the new mechanisms underlying their antiviral potential and cellular functions (Yang et al., 2012).

IFN-induced transmembrane proteins (IFITM)

Next to IFIT proteins, also IFN-induced transmembrane (IFITM) proteins were recently identified as broad-spectrum inhibitors of a range of human viruses. Despite their similar abbreviation, they control virus infections through quite distinct mechanisms. As mentioned above, IFIT proteins suppress translation initiation, bind uncapped or incompletely capped viral RNA, and sequester viral proteins or RNA in the cytoplasm. IFITM proteins, by contrast, interfere with entry of several enveloped viruses (including influenza A virus, HIV-1 and SARS-CoV) by inhibiting viral cytoplasmic release which is characterized by fusion with the endosomal or lysosomal membranes. For both families, additional work is needed to completely unravel their mode of action (Brass et al., 2009; Diamond and Farzan, 2013; Lu et al., 2011a).

Promyelocytic leukaemia nuclear bodies (PML NBs)

The PML gene has been identified more than 20 years ago in acute promyelocytic leukemia (APL). PML protein is a member of the tripartite motif (TRIM) family with a RING (really interesting new gene) domain that contributes to the biological diversity by mediating the conjugation of ubiquitin and ubiquitin-like modifiers such as SUMO (small ubiquitin modifier). It is the organizer of PML NBs, which are small dynamic nuclear substructures that range in size and composition and which recruit, via PML, numerous other transiently or permanently localised IFN-α/β inducible proteins. Functionally, PML NB may sequester, modify or degrade partner proteins through facilitating post-translational modifications, which demonstrates their involvement in a wide range of cellular pathways (including oncogenesis, transcriptional responses to stress, chromatin structure remodelling, apoptosis, ubiquitin pathway) (Everett and Chelbi-Alix, 2007; Lallemand-Breitenbach and de The, 2010). Additionally, they represent one of the IFN protective pathways to impede viral infection, although the exact mechanism has only been elucidated for a few selected viruses, including hepatitis B virus (HBV), human immunodeficiency virus (HIV), herpes simplex virus-1 (HSV-1) and human cytomegalovirus (HCMV) (Gao et al., 2009; Geoffroy and Chelbi-Alix, 2011; Stremlau et al., 2004; Uchil et al., 2008).

Bone marrow stromal antigen 2 (BST-2/tetherin)

BST-2 is another antiviral weapon in the arsenal of the innate immune response which inhibits virus release by tethering virions to the cell surface. It has been identified in the genomes of all mammals analyzed to date. In principal tetherin could target any enveloped virus that buds from cellular membrane enriched in tetherin, but most knowledge has been

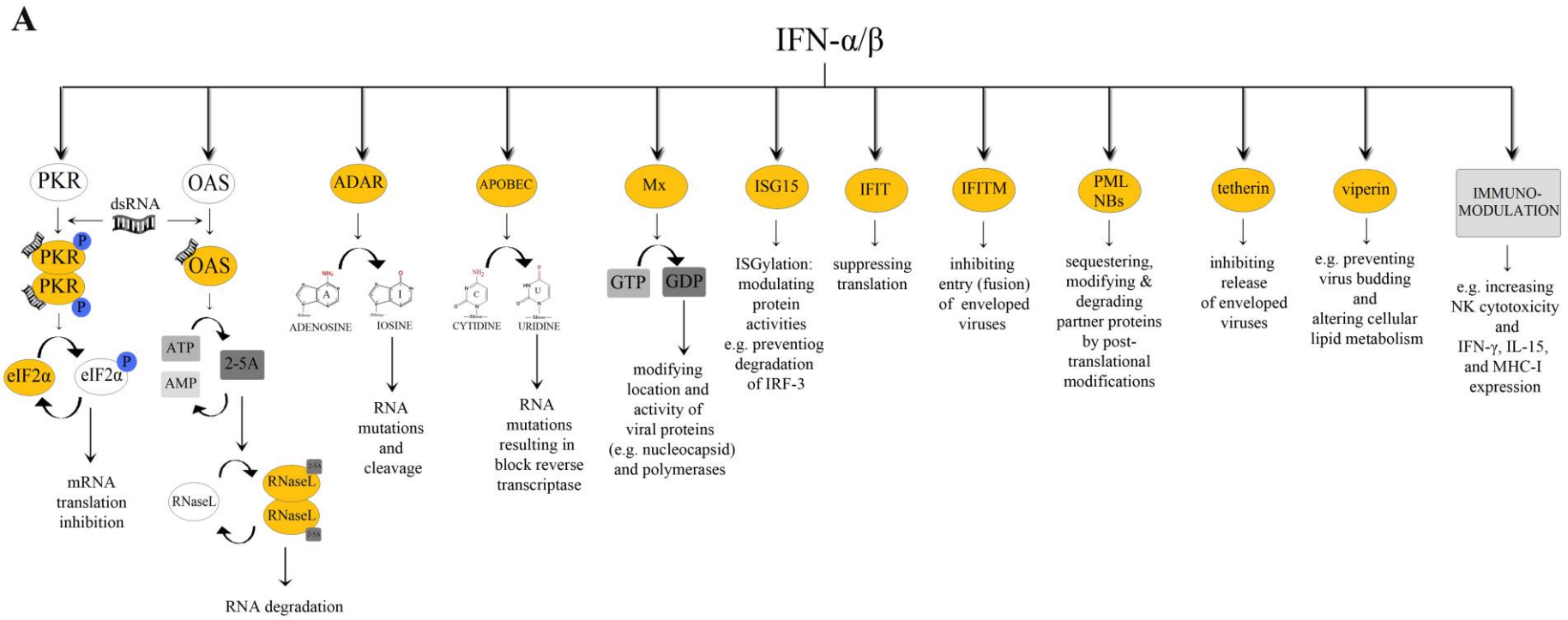
gathered about its powerful action against retroviruses, including HIV-1 (Andrew et al., 2012; Le Tortorec et al., 2011; Neil et al., 2008).

Virus inhibitory protein, ER-associated, IFN-inducible (viperin)

Recent published data indicates that viperin is an ISG that inhibits a broad range of DNA and RNA viruses. Up till now, there is evidence that viperin exerts its effects at later stages of the viral life cycle of influenza (by disrupting lipid rafts to prevent virus budding), and interferes with replication of HCV and HCMV (by altering cellular lipid metabolism) (Chin and Cresswell, 2001; Jiang et al., 2008; Seo and Cresswell, 2013; Wang et al., 2007). Despite the considerable progress that has been made in our understanding on how viperin controls infection, its precise mechanism of action on all of the targeted viruses remains mysterious (Fitzgerald, 2011; Seo et al., 2011).

Immunomodulation

Finally, type I IFNs form an important link between early innate and late adaptive immune responses by their potent immunomodulatory functions (Le Bon and Tough, 2002). The most important immunomodulations will be summarized in this paragraph. First of all, type I IFN can promote IFN-γ expression in T cells, which is a key mediator of virus-specific cellular immunity (Samuel, 2001). Furthermore, all IFNs share the ability to control the upregulate class I major histocompatibility complex (MHC-I) molecules and thereby to enhance the repertoire and quantity of peptides displayed to CD8+ cytotoxic T cells. This increases the recognition and killing of infected cells by the activated CD8+ T cells (Boehm et al., 1997; Goodbourn et al., 2000; Samuel, 2001). In addition, IFNs improve the cytotoxicity of NK cells by upregulating perforin and granzymes (Biron et al., 1999; Bolitho et al., 2007; Reiter, 1993). Finally, IFNs can trigger the induction of IL-15 from APCs, which provides survival signals for memory cytotoxic T cells and NK-cells (Di Sabatino et al., 2011; Goodbourn et al., 2000; Lodolce et al., 2002; Samuel, 2001).


A
IFN-α/β
PKR
dsRNA
PKR
PKR
eIF2α
eIF2α
mRNA translation inhibition
OAS
OAS
ATP
AMP
2-5A
RNaseL
RNaseL
RNaseL
RNA degradation
ADAR
ADENOSINE
IOSINE
RNA mutations and cleavage
APOBEC
CYTIDINE
URIDINE
RNA mutations resulting in block reverse transcriptase
Mx
GTP
GDP
modifying location and activity of viral proteins (e.g. nucleocapsid) and polymerases
ISG15
ISGylation: modulating protein activities e.g. preventiog degradation of IRF-3
IFIT
suppressing translation
IFITM
inhibiting entry (fusion) of enveloped viruses
PML NBs
sequestering, modifying & degrading partner proteins by post-translational modifications
tetherin
inhibiting release of enveloped viruses
viperin
e.g. preventing virus budding and altering cellular lipid metabolism
IMMUNO-MODULATION
e.g. increasing NK cytotoxicity and IFN-γ, IL-15, and MHC-I expression

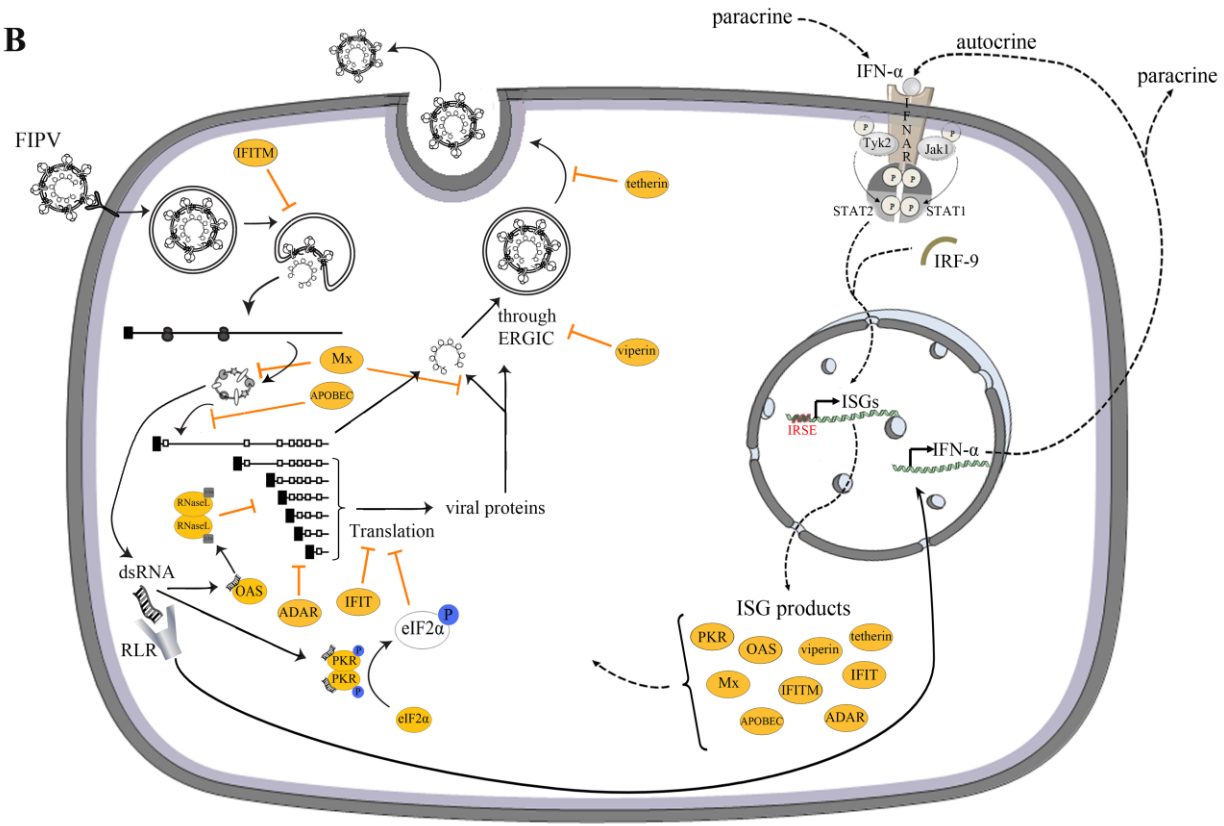


Figure 1.6: (A) Schematic overview of IFN-inducible antiviral proteins and their activities on positive stranded RNA viruses (adapted from Samuel, 2001). (B) Hypothetical model for FIPV induction of IFN-α and JAK/STAT signalling (adapted from Figure 1.5 [p24]) and the intrinsic cellular defences by IFN-α stimulated gene (ISG) products on different steps of the FIPV replication cycle (adapted from Figure 1.4 [p12]).

FIPV enters the cell via endocytosis and after release of its genome, dsRNA is generated as an intermediate of genome replication. The recognition of dsRNA by RIG-I like receptors (RLR) induce the production of IFN-α, which will be released and bind to its receptor (IFNAR) on the infected (autocrine) and neighbouring cells (paracrine). Through the JAK/STAT signalling pathway different ISG products are produced, which may act on FIPV infection: PKR, IFN-induced dsRNA-dependent protein kinase, phosphorylates eukaryotic initiation factor 2α (eIF2α) leading to inhibition of gene translation; RNase L, ribonuclease L, is activated by OAS (oligo-adenylate synthetase) and breaks down viral RNAs; ADAR, Adenosine deaminase acting on RNA, mediates adenosine (A) to inosine (I) conversion in RNA, leading to RNA cleavage or RNA mutations; APOBEC, apolipoprotein B mRNA editing enzyme-catalytic, polypeptide edites cytidine (C) to uridine (U), resulting in both viral genome mutation and inhibition of reverse transcription; Mx, myxovirus resistance, GTPase captures viral nucleocapsid proteins or polymerases to prevent genome replication, transcription or assembly; IFIT, IFN-induced with tetratricopeptide repeats, proteins are important in protein-protein interactions, resulting, among others, in the inhibition of translation; IFITM, IFN-induced transmembrane, proteins interfere with viral membrane fusion during entry of enveloped viruses; tetherin inhibits virus release by tethering virions to the cell surface; viperin alters the cellular lipid metabolism resulting in the disruption of virus budding and replication.

1.3 Coronavirus-encoded countermeasures against type I IFN mediated antiviral responses

The IFN system is an extremely potent antiviral defence against all kinds of viruses. Clearly, an emerging virus must develop efficient escape strategies in order to establish a productive infection. The number of viral IFN antagonistic proteins that are discovered is rapidly growing. These IFN antagonists are often multifunctional proteins that help the virus to cope with the IFN system through (i) interfering with the host protein synthesis; (ii) suppressing IFN production; (iii) blocking IFN signalling or (iv) inhibiting interferon stimulated genes (ISG) antiviral activity. Combined strategies are mostly needed to cover the whole spectrum of the IFN response in an infected cell (Randall and Goodbourn, 2008; Rose and Weiss, 2009). There is substantial evidence that end-balance between virus-promoting and virus-inhibiting factors is greatly dependent on the species and even a specific cell type. One mutation that subtly alters viral fitness in a particular cell type may be enough for the cellular IFN system to gain the advantage (Rose and Weiss, 2009). Numerous studies have contributed to the elucidation of virus encoded IFN antagonizing proteins. Until now, reviews have mainly focussed on the strategies used by vaccinia virus, influenza viruses, herpes viruses, adenoviruses, hepatitis C virus and human immunodeficiency virus. Here, it is summarized how coronaviruses manage to survive in the face of the powerful IFN system (see also Figure 1.7 and Table 1.1). Clearly, many coronaviruses encode multiple IFN antagonists to increase the likelihood that the virus delays the induction of the innate immune responses sufficiently to allow for efficient replication and spread. Viruses that are able to target more than one part of the interferon response are most likely to cause a severe inhibition of interferon, contributing to high pathogenicity. Moreover, different antagonists may function more efficiently in different tissues and host cells that are targeted during infection, or may compensate for each other when a viral evasion protein is mutated or neutralized (Frieman et al., 2008; Kopecky-Bromberg et al., 2007).

1.3.1 Interfering with the host protein synthesis

It has been shown for both SARS-CoV and MHV that their non-structural protein 1 (nsp1) strongly suppresses cellular gene synthesis. Nsp1 promotes host mRNA degradation (including type I IFN mRNA) and inhibits host protein translation, probably through the direct interaction with distinct host factors, like e.g. the 40S subunit of the ribosome (Bechill et al., 2008; Jauregui et al., 2013; Narayanan et al., 2008a; Zust et al., 2007). Although

Wathelet and co-workers also demonstrated a potential IFN inhibiting role for nsp1, they did not observe the RNA degradation phenotype. This indicates that additional work is needed to delineate the exact role of nsp1 (Wathelet et al., 2007). Next to nsp1, also the S protein of SARS-CoV, as well as IBV, is involved in the sustained shutdown of host cell translation. The spike protein was shown to interact with the initiation factor eIF3f, leading to disruption of the organisation and activation of translation. This inhibition occurs at late stages of the virus replication cycle. Consequently, it renders little effect on viral production and spread because the viral RNA replication and protein synthesis are nearly complete. Moreover, the main inhibitory effect would be on the translation of proinflammatory cytokines and chemokines (e.g. IL6 and IL8) which are heavily induced due to infection, resulting in a suppressed inflammation and tissue injury (Xiao et al., 2008).

At first sight, interfering with the global host translation, including IFN production appears to be a very good way for the virus to establish an infection. However, cells in which the cellular protein synthesis is inhibited will die very rapidly, which gives the virus limited time to replicate. Moreover, the virus will not be able to manipulate the cell to its own advantage e.g. providing the enzymes that are needed for virus replication, and persistent or latent infection in those cells will not be possible (Randall and Goodbourn, 2008). Therefore, viruses have evolved other, more subtle means of circumventing the IFN response.

1.3.2 Suppressing IFN production

Virus induced IFN production can depend on several factors including the cell/tissue type, the amount and type of IFN inducers produced by the virus and whether the virus produces a specific inhibitor of the IFN induction pathways. It has been shown that several β-CoV do not efficiently trigger production of IFN, while α-CoV (like HCoV-229E and TGEV) do. This difference has been mapped to the N-glycosylation pattern of the ectodomain of the M protein of α-CoV (de Haan et al., 2003; Laude et al., 1992; Zhou and Perlman, 2007). Nevertheless, the lack of IFN production by β-CoV has not been seen in all infected cell types. In particular, different MHV strains do not induce IFN in fibroblasts and bone-marrow derived DCs, while they do in plasmacytoic DCs and glial cells (Rose and Weiss, 2009; Roth-Cross et al., 2007; Zhou and Perlman, 2007). The level of MDA5 mRNA expression in uninfected cells can be one way to explain the difference in the ability to induce IFN by different cell types (Roth-Cross et al., 2008). The different strategies used by coronaviruses to keep the production of IFN to a minimum are discussed below and comprise the reduction of viral PAMPs exposure and the downregulated activation of TFs needed for IFN induction.

In general, the strategy of coronaviruses to replicate within double-membrane vesicles (**DMVs**), might enable them to shield their viral RNA for cellular PRRs. For MHV, this seems to be directed by nsp4 protein, as its mutation results in aberrant and open vesicles (Clementz et al., 2008). However, such a passive mechanism is not sufficient to completely suppress IFN induction, and therefore CoVs also use other mechanisms to actively inhibit IFN production (Totura and Baric, 2012; Versteeg et al., 2007). Several coronavirus proteins, like N protein of SARS-CoV and accessory protein 4a of MERS-CoV, possess a **dsRNA binding motif.** This enables them to bind to and sequester viral RNAs, which inhibits the IFN production by blocking the RNA-sensor recognition step and subsequent RIG-I and MDA5 activation (Lu et al., 2011b; Niemeyer et al., 2013). The host can distinguish viral non-self mRNAs from self mRNAs due to the lack of a 5' cap. The ability of a virus to mimic this host **capping machinery** or to modify the cap of viral RNAs, enables the virus to evade the recognition by host PRRs. Two proteins of SARS-CoV have been described to have this potential, being nsp14, identified as a guanine-N7-methyltransferase, and nsp16, possessing a 2'-O-methyltransferase activity (Totura and Baric, 2012).

Papain-like protease (PLpro) encoded by nsp3 of three CoVs (HCoV-NL63, SARS-CoV and MHV) has been proven to limit the type I IFN production (Clementz et al., 2010; Devaraj et al., 2007; Frieman et al., 2009; Rose and Weiss, 2009). The exact mechanism has recently been elucidated for SARS-CoV and HCoV-NL63. It was shown that PLpro associates with an ER-associated protein STING (stimulator of interferon genes) and blocks assembly or stability of STING dimers which are important for **IRF-3 nuclear translocation** through the MAVS signalling pathway (Sun et al., 2012). Additionally, PLpro is able to block **NF-κB signalling** through the stabilization of the NF-κB inhibitor iκBα (Frieman et al., 2009). In SARS-CoV and Bat SARS-like CoV, the accessory ORF3b has also been ascribed as an inhibitor of the IFN synthesis, by blocking the IRF-3 nuclear translocation (Kopecky-Bromberg et al., 2007; Zhou et al., 2012). It was suggested that ORF3b may inhibit MAVS at the mitochondria or that it may function as a virally encoded chaperone that binds and removes host-encoded proteins (like IRF-3) from the nucleus (Freundt et al., 2009). Another accessory ORF of SARS-CoV that has been identified as an inhibitor of IRF-3 phosphorylation and nuclear translocation is ORF6. Moreover, ORF6 protein was able to reduce the expression of the NF-κB responsive promoter, probably by binding to the nuclear import cargo of NF-κB (Hussain and Gallagher, 2010; Kopecky-Bromberg et al., 2007). Last but not least, SARS-CoV M protein inhibits type I IFN production by binding and sequestering TRAF3, RIG-I, TBK1 and IKKε in the cytoplasm. Thereby, it impedes the

formation of a TRAF3-TANK-TBK1/IKKε complex, which is needed to phosphorylate and subsequent activate IRF-3/IRF-7 transcription factors (Siu et al., 2009).

1.3.3 Blocking IFN signalling

It has become clear that some viral proteins can exert multiple different antagonistic effects on the IFN system. As mentioned above, nsp1 and PLpro of SARS-CoV have been identified as strong inhibitors of host protein synthesis or IFN production, respectively, but they can both also function as an inhibitor of IFN signalling, by specifically decreasing the phosphorylation levels of **STAT1** (Li et al., 2011a; Wathelet et al., 2007). It was elucidated that PLpro can suppress this STAT1 phosphorylation by degradation of ERK1 via the activation of ubiquitin proteasome pathways (Li et al., 2011a). Furthermore, accessory ORF3b and ORF6 are also capable of blocking ISRE promoter expression, next to their capacity of inhibiting IFN production (Kopecky-Bromberg et al., 2007). ORF6 interacts via its C-terminal region to the nuclear import protein karyopherin alpha 2 (KPNA2), resulting in the tethering of KPNA2 to the ER/Golgi membrane. In this way, ORF6 protein is indirectly interfering with STAT1 nuclear translocation because KPNA2 functions as an import cargo for STAT1 (Frieman et al., 2007; Hussain and Gallagher, 2010). The nsp2 of MHV is partially **inhibiting the induction of ISGs like ISG15 and IFIT** but its exact mechanism has not been described. It seems that its ability to antagonize IFN responses is cell-type specific, as it is necessary for bone-marrow derived macrophage (BMM) replication and the subsequent liver tropism and hepatitis induction but dispensable for pathogenesis in the central nervous system. Probably, the high basal expression levels of ISGs in BMM are explaining the liver-specificity of this virulence factor (Zhao et al., 2011).

Another mechanism that can be used by viruses to block the IFN signalling, is by reducing the levels of IFN receptors at the cell surface. This phenomenon has been described in cells expressing accessory protein 3a of SARS-CoV. This protein leads to the serine phosphorylation of **IFNAR1 degradation** motif, resulting in an increased IFNAR1 ubiquitination and lysosomal degradation (Minakshi et al., 2009).

1.3.4 Inhibition of ISG antiviral activity

For both MHV (β-CoV) and IBV (γ-CoV), nsp2 was identified as a strong IFN antagonist by acting on the activity of ISGs. Nevertheless, their regulation mechanism was not really comparable. Nsp2 of IBV acts as a **PKR antagonist** by inducing the overexpression of Growth Arrest and DNA Damage-Inducible Protein (GADD34) which participates in the

eIF2α dephosphorylation, thereby reversing the action of PKR and ensuring sustained viral mRNA translation (Wang et al., 2009). On the other hand, nsp2 of MHV has a 2'-5'-phosphodiesterase activity and can cleave 2-5A, thereby **inhibiting RNaseL activation** and consequently blocking viral RNA degradation (Zhao et al., 2012). But nsp2 is not the only protein of MHV that is functioning on this pathway. Providing N protein of MHV to the highly IFN-sensitive virus VVΔE3L (vaccinia virus lacking E3L) inhibited RNA degradation and partly restored translation. It was shown that N protein has RNA binding properties, and that it possibly could prevent dsRNA-dependent activation of OAS and probably PKR, by sequestering or masking dsRNA from these enzymes (Ye et al., 2007). A third protein that has been identified as a major component that determines the IFN resistance of MHV, is the accessory protein 5a. Although its exact mechanism has not been elucidated, it is clear that it does not mediate its antagonistic effect through interaction with either the PKR or OAS antiviral system (Koetzner et al., 2010). The opportunity to interfere with important antiviral ISG activities was also demonstrated for an α-CoV, namely TGEV. Deleting TGEV encoded accessory protein 7, which has a high similarity with protein 7a of other genus α1 CoVs, resulted in increased interferon and proinflammatory responses both *in vitro* and *in vivo* (Cruz et al., 2013). It was proven that protein 7 interacts with PP1c and modulates/directs its activity to both PKR and OAS/RNaseL pathways. This may counteract PKR as well as RNaseL activation through the dephosphorylation of eIF2α and 2-5A, respectively. Gene 7 might counteract host defenses with the aim of preventing overwhelming tissue damage due to an exacerbated innate immune response. In that way, protein 7 would benefit both the host, reducing pathology caused by the infection, and the virus, allowing longer virus persistence and dissemination (Cruz et al., 2011). In SARS-CoV infected cells a sustained PKR activation and eIF2α phosphorylation was detected but this could not impair virus replication, suggesting that SARS-CoV possesses a mechanism to overcome the inhibitory effects of P-eIF2α on viral mRNA translation (Krahling et al., 2009).

Controlling protein synthesis (by antagonizing PKR and OAS) is one of the most important antagonistic effects of viruses, as they fully rely on the canonical cellular translation machinery to translate their own RNAs. However, counteraction with other ISGs was also demonstrated for a number of viral proteins, including Vaccinia virus E3L protein (targeting ADAR), Influenza B virus NS1 protein (targeting ISG15), HSV-1 ICP0 protein (targeting PML), HCV core protein (targeting PML), HIV vif protein (targeting APOBECs) (Boutell et al., 2002; Conticello et al., 2003; Herzer et al., 2005; Liu et al., 2005; Liu et al., 2001; Yuan

and Krug, 2001). Nevertheless, until now, no research data are available on coronaviral evasion proteins that could interfere with ISGs other than PKR and OAS.

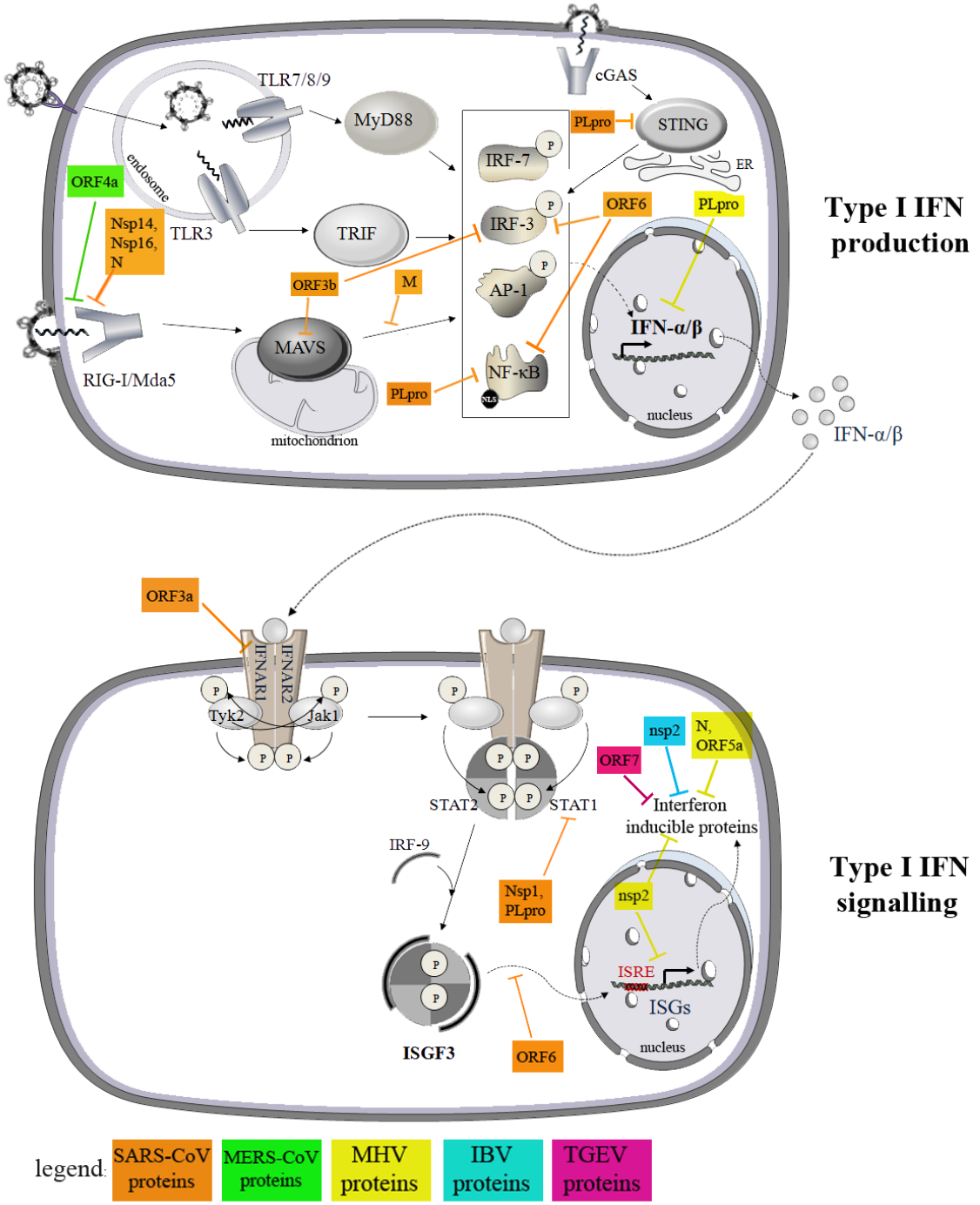


Figure 1.7: Intrinsic cellular defences of viral IFN antagonists encoded by coronaviruses.

Virus	Antagonistic activity on …	Protein	Mode of action	References
SARS-CoV	host protein synthesis	nsp1	inhibits mRNA translation and induces mRNA degradation	Narayanan *et al.*, 2008a; Jauregui *et al.*,2013 Wathelet *et al.*, 2007
		S	inhibits mRNA translation by interaction with eIF3f	Xiao *et al.*, 2008
	IFN production	PLpro	blocks IRF-3 translocation by association with STING	Sun *et al.*, 2012
		nsp14 & nsp16	modulates viral RNA capping	Totura and Baric, 2012
		ORF3b	blocks IRF-3 translocation through binding	Freundt *et al.*, 2009
		ORF6	blocks NF-kB signaling through binding with import cargo	Hussain and Gallagher, 2010; Kopecky-Bromberg *et al.*, 2007
		N	binds dsRNA	Lu *et al.*, 2011b
		M	blocks IRF-3/IRF-7 activation by destabilizing TRAF3-TBK1/IKKe	Siu *et al.*, 2009
	IFN signalling	nsp1	decreases STAT1 phosphorylation	Wathelet *et al.*, 2007
		PLpro	decreases STAT1 phosphorylation by degrading ERK1	Li *et al.*, 2011
		ORF3a	induces degradation of IFNAR1	Minakshi *et al.*, 2009
		ORF6	blocks STAT1 translocation by binding to KPNA2	Frieman *et al.*, 2007, Hussain and Gallagher, 2010
MERS-CoV	IFN production	ORF4a	binds dsRNA	Niemeyer *et al.*, 2013
MHV	host protein synthesis	nsp1	inhibits mRNA translation	Bechill *et al.*, 2008; Züst *et al.*, 2007
	IFN production	PLpro	impedes IFN induction by its deubiquitinase activity	Rose and Weiss, 2009
	IFN signalling	nsp2	suppresses ISG15 and IFIT expression	Zhao *et al.*, 2011
	ISG activity	nsp2	inhibits RNaseL activation by cleaving 2-5A	Zhao *et al.*, 2012
		N	inhibits OAS and PKR activation by binding dsRNA	Ye *et al.*, 2007
		ORF5a	?	Koetzner *et al.*, 2010
IBV	host protein synthesis	S	inhibits mRNA translation by interacting with eIF3f	Xiao *et al.*, 2008
	ISG activity	nsp2	counteracts PKR activity by overexpression of GADD34	Wang *et al.*, 2009
TGEV	ISG activity	ORF7	counteracts OAS and PKR activation by recruiting PP1c	Cruz *et al.*, 2011; Cruz *et al.*, 2013

Table 1.1: Overview of coronaviruses and their viral proteins that counteract IFN-mediated antiviral effects

1.4 References

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Chapter 2

Aims

Feline infectious peritonitis virus is the causative agent of a highly fatal disease in cats, designed as FIP. The disease is characterized by replication in monocytes/macrophages, which induces a systemic invasion of the host followed by an intense inflammation reaction in affected tissues. Until now, the pathogenesis has not yet been fully elucidated, which hampers early diagnosis and the development of efficient treatments or vaccines.
During evolution, a high selective pressure enforces the viruses to find the ideal balance between the number of viral proteins needed for efficient host infection and the high investment cost to express these proteins. This means that viruses will only preserve the genes that eventually give them the highest fitness. Therefore, the maintenance of ORFs encoding for accessory proteins in the feline coronavirus genome, has stirred up our interest. There are several indications that these group-specific genes are of crucial importance in the development of FIP. The general aim of this doctoral research consisted of identifying biological functions of accessory proteins encoded by FIPV. Targeting these proteins, and consequently their functions, could provide us new strategies to treat FIP.

The first study focused on the importance of the accessory proteins for the replication of FIPV in its target cell, the feline monocytes (**Chapter 3**).

During many infections, especially with RNA viruses, the host cells activate an antiviral state, controlled by type I interferon induction. In order to establish an efficient replication in host cells, viruses must evade this immune response. Many viruses are using non-structural or accessory proteins for this purpose, which are otherwise non-essential for virus growth. Therefore, it was investigated if FIPV infections are also inducing this antiviral IFN response and if the virus was able to counteract this response. Furthermore, the involvement of FIPV accessory proteins in reducing virus sensitivity towards IFN-α was determined (**Chapter 4**).

Finally, the IFN-counteracting mechanism was examined in more detail, focussing on the specific antagonistic properties of FCoV accessory protein 7a **(Chapter 5)**.

Chapter 3

The Role of Accessory Proteins in the Replication of Feline Infectious Peritonitis Virus in Peripheral Blood Monocytes

Adapted from:

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Abstract

The ability to productively infect monocytes/macrophages is the most important difference between the low virulent feline enteric coronavirus (FECV) and the lethal feline infectious peritonitis virus (FIPV). *In vitro*, the replication of FECV in peripheral blood monocytes always drops after 12 hours post inoculation, while FIPV sustains its replication in 45% of the cats. The accessory proteins of feline coronaviruses have been speculated to play a prominent role in virulence as deletions were found to be associated with attenuated viruses. Still, no functions have been ascribed to them. In order to investigate if the accessory proteins of FIPV are important for sustaining its replication in monocytes, replication kinetics were determined for FIPV 79-1146 and its deletion mutants, lacking either accessory protein open reading frame 3abc (FIPV-Δ3), 7ab (FIPV-Δ7) or both (FIPV-Δ3Δ7). Results showed that the deletion mutants FIPV-Δ7 and FIPV-Δ3Δ7 could not maintain their replication, which was in sharp contrast to wt-FIPV. FIPV-Δ3 could still sustain its replication, but the percentage of infected monocytes was always lower compared to wt-FIPV. In conclusion, this study showed that ORF7 is crucial for FIPV replication in monocytes/macrophages, giving an explanation for its importance *in vivo*, its role in the development of FIP and its conservation in field strains. The effect of an ORF3 deletion was less pronounced, indicating only a supportive role of ORF3 encoded proteins during the infection of the *in vivo* target cell by FIPVs.

3.1 Introduction

Feline coronaviruses (FCoVs) are enveloped viruses with a large positive-stranded RNA genome of about 30 kilobases (Dye and Siddell, 2005). The 5' two-thirds of the genome encodes for polypeptides that are subsequently cleaved by viral proteinases to yield 16 functional proteins, mainly involved in viral RNA synthesis. The proteins on the 3'-proximal region of the genome are individually expressed from a nested set of subgenomic mRNAs generated by a unique discontinuous transcription mechanism (Enjuanes et al., 2006). These mRNAs cover the four structural proteins; the spike (S), nucleocapsid (N), membrane (M) and envelope (E) proteins, and five accessory proteins 3a, 3b, 3c, 7a and 7b which are most likely produced by leaky scanning from two gene cluster open reading frames ORF3 and ORF7 (Schaecher et al., 2007).

Based on virus antigenicity, two serotypes can be distinguished among FCoVs. Type I feline coronaviruses are most commonly found in the field (Hohdatsu et al., 1992; Vennema, 1999). Type II feline coronaviruses originate from a double recombination between type I feline coronavirus and canine coronavirus (CCV) (Herrewegh et al., 1998). Until now, most experiments have been performed with type II viruses because type I viruses are difficult to grow *in vitro*. Both serotypes contain two pathotypes. The low virulent feline enteric coronavirus (FECV) is endemic in the cat population and causes mostly asymptomatic infections. In young kittens, it can cause mild gastrointestinal infections (Pedersen et al., 1981b). The highly pathogenic feline infectious peritonitis virus (FIPV) manifests in a progressive and mostly fatal disease, FIP, characterized by granulomatous lesions (Egberink et al., 1995; Pedersen, 2009). The most prominent factor that determines the difference between the two pathotypes is the ability to establish an efficient and productive infection in monocytes/macrophages (Dewerchin et al., 2005; Rottier et al., 2005). The low virulent FECV mainly infects enterocytes but can also enter and replicate in monocytes (Desmarets et al., 2013; Dewerchin et al., 2005; Meli et al., 2004). However, it was shown *in vitro* that after one replication cycle, the percentage of FECV infected monocytes decreases (Dewerchin et al., 2005). This is probably the reason why only low viral loads of FECV can be found systemically and infection does not lead to FIP. In contrast, the highly pathogenic counterpart, FIPV, has the ability to productively infect monocytes and this sustained infection is probably the basis for the development of FIP (Kipar et al., 2006).

RNA viruses, such as FCoVs, generally have very high mutation rates compared to DNA viruses, because viral RNA polymerases lack proof-reading. This, together with the close

genetic similarity of FECV to FIPV and the low incidence of FIP, despite the high prevalence of FCoV seropositive cats, led to the widespread acceptance of the "internal mutation theory". Numerous publications supported that FIPV, unlike FECV, is not transmitted from cat to cat but arises from FECV by mutation within an individual cat (Chang et al., 2010; Foley et al., 1997; Herrewegh et al., 1995; Pedersen et al., 2012; Rottier et al., 2005; Vennema et al., 1998). Up till now, it is not yet completely clarified what these FIP-inducing mutations in FECV might be. The C-terminal domain of the FIPV spike protein has already been shown to be important for efficient macrophage infection (Chang et al., 2012; Rottier et al., 2005). In addition, accessory proteins have been proposed to be potential targets for mutations and as such important for virulence. These accessory proteins are species-specific and are dispensable for *in vitro* replication. During passaging of CoVs in cell cultures, viral mutants with deletions in accessory ORFs arise spontaneously and show growth advantages (Lorusso et al., 2008), suggesting that loss of accessory gene expression increases viral fitness *in vitro*. Despite their redundancy *in vitro*, deletion of accessory ORF clusters from FIPV 79-1146, mouse hepatitis virus (MHV) and transmissible gastroenteritis virus (TGEV) genome resulted in attenuated viruses (de Haan et al., 2002; Haijema et al., 2004; Ortego et al., 2003). Thus, these proteins are of key importance for virus-host interactions and critically contribute to viral virulence and pathogenesis. Nevertheless, a clear function could not be attributed so far to any of the accessory proteins of FCoVs.

Based on their sequence, both 3a and 3b proteins are predicted to consist of 72 amino acids (AA). None of these predicted proteins have a hydrophobic segment that can serve as a signal peptide or transmembrane domain, so they are probably located in the cytosol where they exert an intracellular function. Their sequences are well conserved within the two pathotypes, while more differences have been seen between the two serotypes (Volker, 2007). Comparative sequence analysis with CCV and FCoV strains revealed that type II FCoV has received these genes from CCV.

Translating the consensus nucleotide sequence, the 3c protein appears to be 238 AA in length. Hydrophobicity analysis predicts that 3c is a class III triple-membrane spanning protein suggesting a topology that is quite similar to the one of the M protein of FCoVs and the 3a protein of the 'severe acute respiratory syndrome' coronavirus (SARS-CoV) (Narayanan et al., 2008b; Oostra et al., 2006). In the first quarter of the amino acid sequence of the 3c gene, big differences are observed between the two serotypes. Again the double recombination between type I FCoV and CCV could explain these differences. Remarkably, in studies of Pedersen et al. (2012) and Chang et al. (2010) 60-71.4% of FIP isolates, had a

truncated 3c protein, while in FECV isolates from feces this protein was intact. Chang and colleagues launched the hypothesis that 3c could be necessary for replication in enterocytes but dispensable or even a burden during FIPV infection in monocytes. This may also explain the restricted transmission of FIPV from cat to cat. A recent study added to these findings that a truncated ORF3 was important for efficient *in vitro* replication of FIPV type II in feline monocytes (Balint et al., 2012).

The 7a protein, a small hydrophobic protein of 101 AA (~10kDa) with a N-terminal cleavable signal sequence and a C-terminal transmembrane domain, is more or less well conserved among FCoVs (Volker, 2007). It has a 72% homology with TGEV protein 7 which has been demonstrated to function against host antiviral responses (Cruz et al., 2011).

Most knowledge has been gathered about the 7b protein, which is a soluble glycoprotein of ~24 kDa (206 AA). A lot of single amino acid polymorphisms are present between FCoV strains. Antibodies against 7b protein, found in serum of FECV-seropositive and FIP cats, imply expression of this protein during natural infections (Kennedy et al., 2008). It contains a KDEL-like endoplasmic reticulum (ER) retention signal (=KTEL) at its C-terminus (Vennema et al., 1992). The 7b protein is initially retained in the ER, whereupon it is secreted from infected cells. As 7b is released extracellular, it might function as a virokine which acts as an immune-modulator of host immune responses (Herrewegh et al., 1995; Rottier, 1999). Again the real function has not yet been elucidated.

Knowing that deletion of accessory genes is associated with attenuated viruses (Haijema et al., 2004) and only virulent FIPVs can sustain its replication in monocytes (Dewerchin et al., 2005), the hypothesis was created that one or more accessory proteins may be needed by FIPV for productive replication in feline monocytes. In the present study, this hypothesis was investigated by establishing *in vitro* replication kinetics of FIPV 79-1146 and its accessory ORF deletion mutants in feline peripheral blood monocytes. Results were compared with kinetics in fcwf cells, which is one of the often used model cell lines for infection studies of type II FCoVs. In this way, the function of the accessory proteins was evaluated in its natural host cell.

3.2 Materials and methods

3.2.1 Cats

Three purpose-bred FCoV, FeLV and FIV antibody-negative cats were used as blood donors for the infection kinetics study. The cats were maintained in a temperature-controlled closed

household (approved by Local Ethical Committee).

3.2.2 Viruses and cells

Crandell Rees feline kidney (CrFK) cells were used to obtain third passages of type II FIPV strain 79-1146. FIPV strain 79-1146 was kindly provided by Dr. Rottier (Faculty of Veterinary Medicine, Utrecht University, The Netherlands)

Felis catus whole fetus (fcwf) cells were used to obtain fifth passages of FIPV-Δ3, FIPV-Δ7 and FIPV-Δ3Δ7. These three viruses are deletion mutants from type II FIPV strain 79-1146, kindly provided by Dr. Rottier (Faculty of Veterinary Medicine, Utrecht University, The Netherlands). They are respectively deleted in open reading frames (ORF) 3abc, 7ab or both 3abc and 7ab, using reverse genetics (Haijema et al., 2004).

Blood monocytes were isolated from feline blood and seeded on glass coverslips in a 24-well dish, as described previously (Dewerchin et al., 2005).

3.2.3 Inoculation of fcwf cells and monocytes

Forty-five hours after seeding, fcwf cells and feline monocytes were inoculated with FIPV 79-1146, FIPV-Δ3, FIPV-Δ7 and FIPV-Δ3Δ7 at a multiplicity of infection (m.o.i.) of 0.02. After 1h incubation at 37°C with 5% CO_2, cells were washed 3 times with RPMI-1640 and further incubated in medium.

3.2.4 Detection of viral nucleocapsid protein expression in FCoV infected monocytes and fcwf cells

Monocytes or fcwf cells were fixed at 0, 6, 12 and 24 hours post inoculation (hpi) with 4% paraformaldehyde and further permeabilized with 0.1% Triton X-100 (Sigma-Aldrich GmbH, Steinheim, Germany). To identify FCoV-infected cells, viral cytoplasmic antigens were stained with mouse IgG1 monoclonal antibody (mAb) 10A12 (produced and characterized in our laboratory) which recognizes the nucleocapsid (N) protein, followed by fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (Molecular Probes). Nuclei were visualized with Hoechst 33342 (Molecular Probes). Finally, glass coverslips were mounted on microscope slides using glycerin-PBS solution (0.9:0.1, vol/vol) with 2.5% 1,4-diazabicyclo(2,2,2)octane (Janssen Chimica, Beerse, Belgium). The percentage of infected cells at each time point and for every virus was determined by fluorescence microscopy (Leica Microsystems DMRBE).

3.2.5 Growth curves of feline coronaviruses in fcwf cells and monocytes

At different time points post inoculation, culture medium was harvested and centrifuged at 400xg for 10 minutes. Supernatant was stored at -70°C until titration of extracellular virus. Intracellular virus was obtained by scraping the remaining cells in 1ml fresh medium. Cells were transferred to the eppendorf with the pellet of the centrifuged extracellular virus and subjected to two freeze-thaw cycles. Virus titres were assessed by a 50% tissue culture infective dose assay using fcwf cells. The 50% end-point was calculated according to the method of Reed and Muench (Reed and Muench, 1938).

3.2.6 Statistical analysis

Statistical analysis was performed by Mann-Whitney U tests with SPSS 19.0 (SPSS Inc., Chicago, Illinois, USA). Significant differences were considered if $p \leq 0.1$ (Noymer, 2008).

3.3 Results

3.3.1 Viral nucleocapsid protein expression kinetics in FCoV-infected fcwf cells

After infection of fcwf cells at a m.o.i. of 0.02, viral antigen positive cells were first detected at 6hpi and the percentage kept increasing afterwards (Figure 3.1). FIPV always gave the highest percentage of infected fcwf cells at 24hpi (56.6 ± 15.6 %), followed in order by FIPV-Δ3 (41.5 ± 12.3%), FIPV-Δ7 (37.0 ± 11.4%) and FIPV-Δ3Δ7 (18.2 ± 7.6%)
Figure 3.1 and Figure 3.2). At that time point, the percentage of fcwf cells infected with FIPV-Δ7 and FIPV-Δ3Δ7 was significantly lower compared to wt-FIPV indicating a role of ORF7 during FIPV replication.

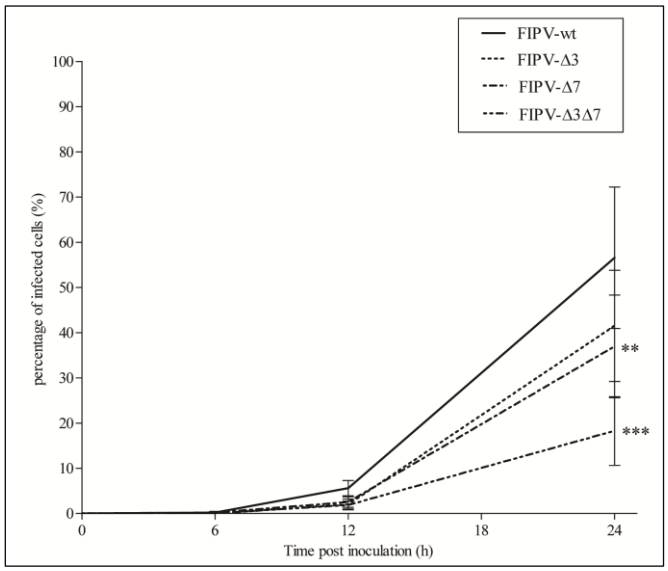

Figure 3.1: Kinetics of percentage of infected cells in FCoV inoculated fcwf cells. Cells were inoculated with FIPV 79-1146, FIPV-Δ3, FIPV-Δ7 and FIPV-Δ3Δ7 at a m.o.i. of 0.02. At different time points post inoculation, cells were fixed and cytoplasmic nucleocapsid protein was visualized with an immunofluorescence staining. The data represent means ± SD of five replicate assays. Significant difference with FIPV 79-1146 is indicated with asterisk * ($p \leq 0.1$), ** ($p \leq 0.05$) or *** ($p \leq 0.01$)

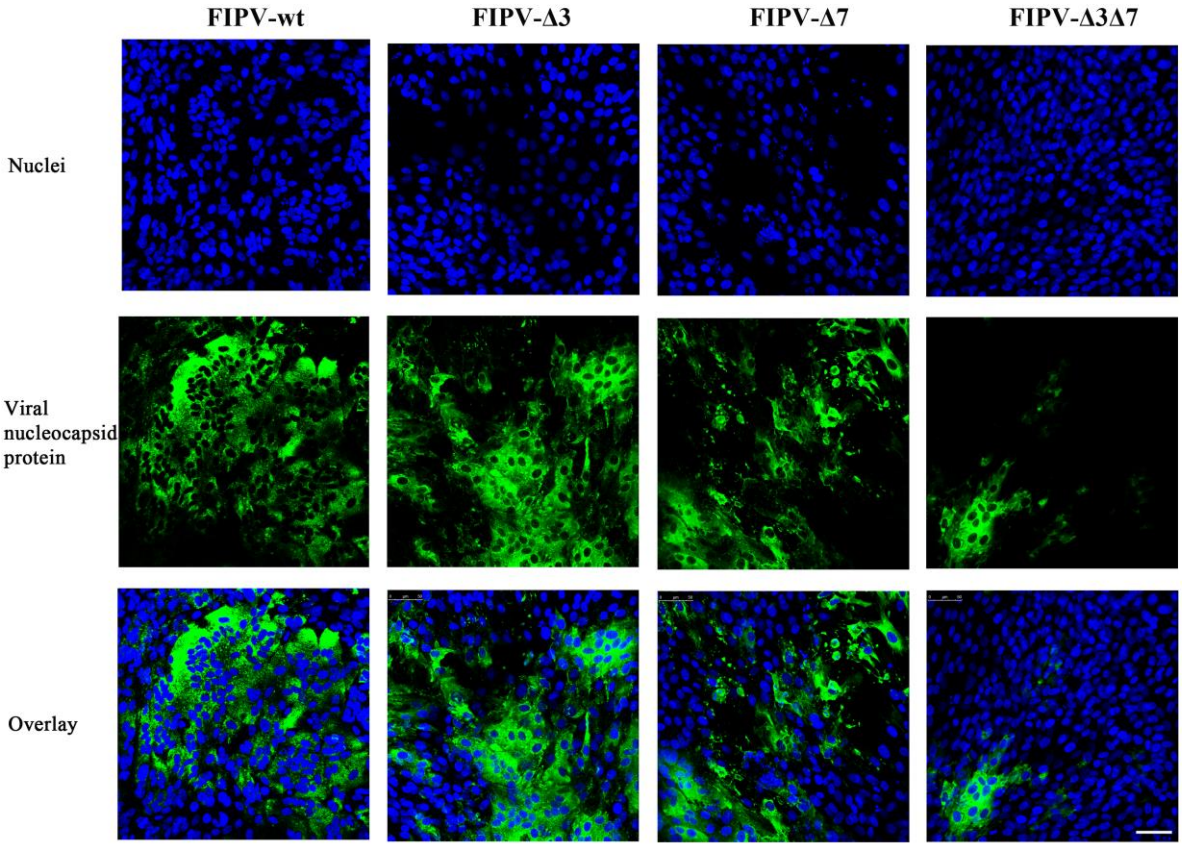

Figure 3.2: Confocal microscopy images of cytoplasmic expression of the nucleocapsid proteins (green) in fcwf cells 24 hours after inoculation with FIPV 79-1146, FIPV-Δ3, FIPV-Δ7 and FIPV-Δ3Δ7 at a m.o.i. of 0.02. Nuclei were visualized with Hoechst 33342 (blue). Bar = 50µm

3.3.2 Growth kinetics of FCoV in fcwf cells

Growth curves for the different viruses in fcwf cells are given in Figure 3.3. Production of progeny virus was detected between 6 and 12hpi and kept increasing up till 24hpi. Concerning the intracellular titre, the increase between 12 and 24hpi was significant stronger for FIPV 79-1146 and FIPV-Δ3 (up to 7.35 $\log_{10}$ $TCID_{50}/10^6$ cells at 24hpi) than for FIPV-Δ7 and FIPV-Δ3Δ7 (up to 5.7 and 5.1 $\log_{10}$ $TCID_{50}/10^6$ cells, respectively, at 24hpi). The extracellular virus titres showed a similar pattern with slightly lower titres for FIPV 79-1146 and FIPV-Δ3 (up to 6.8 $\log_{10}$ $TCID_{50}/10^6$ cells at 24hpi). FIPV-Δ7 and FIPV-Δ3Δ7 reached the same extracellular as intracellular titres.

The amount of infectious virus assembled per cell was calculated based on virus titres and percentages of infected cells. All viruses formed a similar low amount of infectious viruses per infected cell (less than 5) at 12hpi.

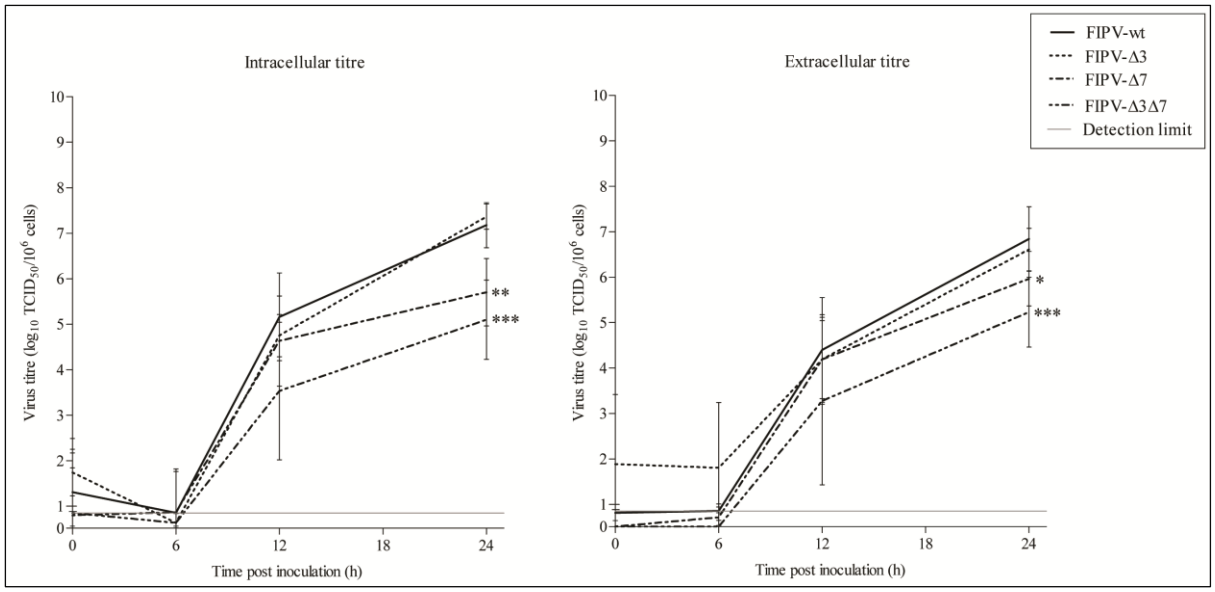

Figure 3.3: Kinetics of FCoV replication in fcwf cells. Cells were inoculated with FIPV 79-1146, FIPV-Δ3, FIPV-Δ7 and FIPV-Δ3Δ7 at a m.o.i. of 0.02. At different time points post inoculation, the intracellular and extracellular virus titres were determined. The data represent means ± SD of five replicate assays. Significant difference with FIPV 79-1146 is indicated with * ($p<0.1$), ** ($p<0.05$) or *** ($p<0.01$).

3.3.3 Viral nucleocapsid protein expression kinetics in FCoV-infected monocytes

The first viral antigen positive monocytes appeared around 6hpi (Figure 3.4). All three cats showed an increase in percentage of infected cells for FIPV 79-1146 up till 24hpi. When ORF3 was deleted from this virus, it was still able to produce infectious virus which kept on infecting exponentially new cells up till 24hpi, though, the percentage of infected cells was 5-20 times lower in comparison with wt-FIPV. The observed difference in percentage infected monocytes between wt-FIPV and FIPV-Δ3 was more pronounced than the difference in percentage infected fcwf cells (which was only 1.3 times).

More remarkable were the results when ORF7 (FIPV-Δ7), or both ORF3 and ORF7 (FIPV-Δ3Δ7) were deleted from FIPV. These mutant viruses initially infected the monocytes similarly as wt-FIPV but from 12hpi the percentage of infected cells declined. Although FIPV-Δ7 and FIPV-Δ3Δ7 reached also a lower percentage of infected cells in fcwf at 24hpi compared to wt-FIPV (1.5 and 3.3 times less, respectively), the difference in infected cells was far more prominent in monocytes (for both deletion mutants 100-3000 times less infected cells in comparison with wt-FIPV).

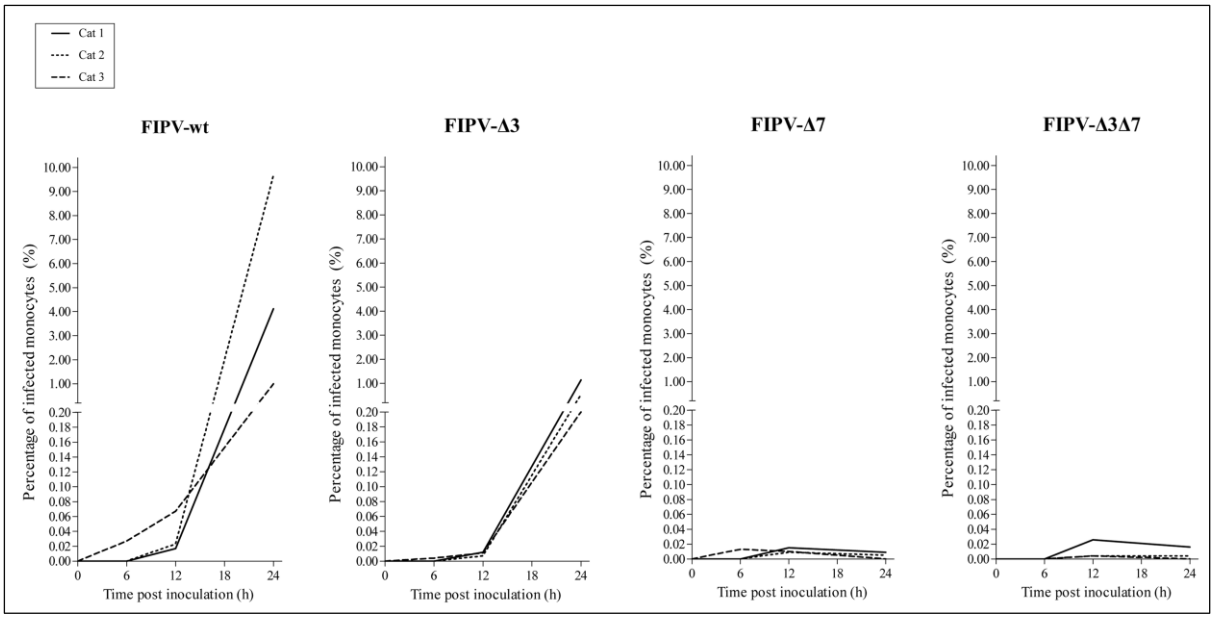

Figure 3.4: Kinetics of percentage of infected cells in FCoV inoculated monocytes isolated from the blood of three independent cats. Cells were inoculated with FIPV 79-1146, FIPV-Δ3, FIPV-Δ7 and FIPV-Δ3Δ7 at a m.o.i. of 0.02. At different time points post inoculation, cells were fixed and cytoplasmic nucleocapsid protein was visualised with an immunofluorescence staining.

3.3.4 Growth kinetics of FCoV replication in feline monocytes

Both wt-FIPV and FIPV-Δ3 kept producing new progeny virus between 12 and 24hpi in monocytes of all three cats but virus titres were always higher for wt-FIPV. In contrast, growth curves of both FIPV-Δ7 and FIPV-Δ3Δ7 reached a plateau or even decreased from 12hpi (Figure 3.5). Knowing the total production of infectious progeny virus and the number of infected cells, it can be calculated that wt-FIPV infected monocytes have produced 50-250 infectious viruses per cell, at 12hpi. Monocytes infected with the three deletion mutants produced a somewhat lower amount of infectious viruses per infected cell (FIPV-Δ3: 4-170 viruses/cell; FIPV-Δ7: 4-50 viruses/cell and FIPVΔ3Δ7: 1-90 viruses/cell).

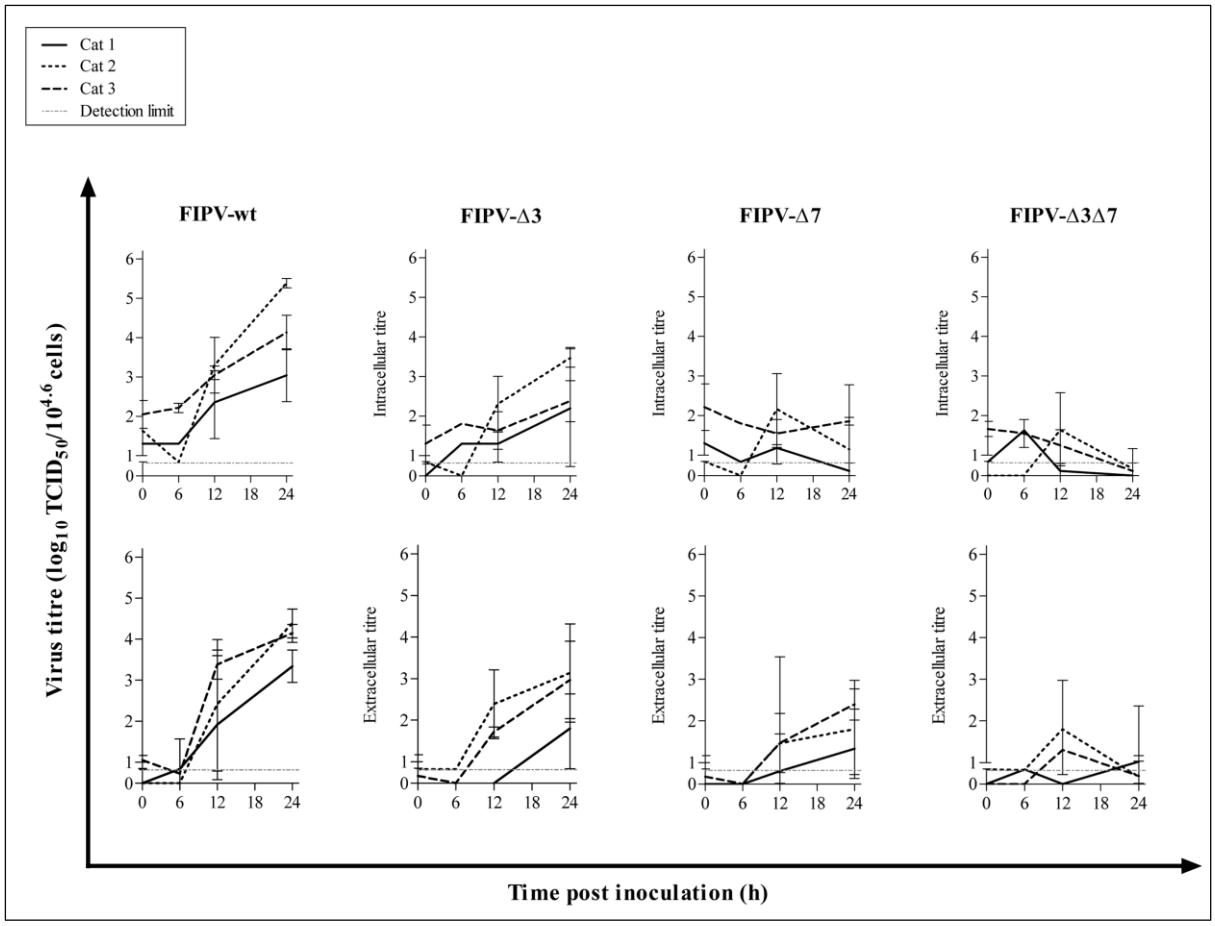

Figure 3.5: Kinetics of FCoV replication in feline monocytes of three cats. Cells were inoculated with FIPV 79-1146, FIPV-Δ3, FIPV-Δ7 and FIPV-Δ3Δ7 at a m.o.i. of 0.02. At different time points post inoculation, intracellular and extracellular virus titres were determined. The data represent means ± SD of triplicate assays.

3.4 Discussion

In the present article, the role of both accessory ORF encoded proteins of FIPV in the replication in its *in vivo* target cell, the feline blood monocyte, was investigated for the first time. This was done by comparing *in vitro* replication kinetics of different accessory gene-deletion mutants of FIPV strain 79-1146 with the kinetics of wt-FIPV in peripheral blood monocytes from three cats. All the viruses were able to initially infect monocytes but only wt-FIPV and FIPV-Δ3 sustained their replication while the replication kinetics of FIPV-Δ7 and FIPV-Δ3Δ7 dropped after one cycle.

Our results indicate that newly produced viruses deleted in ORF7 were not able to enter new monocytes or that virus protein synthesis was inhibited (by an antiviral response) in monocytes cultured for some time in an infected environment. Because currently, accessory proteins are not found in the envelope of feline coronaviruses, it is unlikely that they play a role during binding with and entry of the target cell. Counteraction on the antiviral response

is a far more plausible function for proteins 7a and/or 7b. When cells are infected, they can warn neighbouring cells of viral presence by releasing interferon (IFN). This induces production of several antiviral proteins that have roles in combating viruses or blocking protein synthesis in response to new viral infection (Goodbourn et al., 2000). As ORF7 is located at the 3' far end of the viral genome, where transcription starts (Dye and Siddell, 2005), 7a and 7b mRNA is produced at the very beginning of replication, indicating an early translation of proteins 7a and 7b. It is likely that these proteins interfere quickly with one or more effects of IFN in newly infected cells and as such inhibiting the antiviral response. As a consequence, viruses deleted in ORF7 may not counteract IFN induced antiviral responses anymore and virus replication may be blocked. The crucial role of ORF7 in FIPV replication in monocytes *in vitro*, as shown in our study, could also give an explanation for the attenuated phenotype of ORF7 deletion mutants *in vivo,* observed by Haijema et al. (2004).

In contrast to ORF7 proteins, ORF3 proteins were less important for virus replication in monocytes because FIPV-Δ3 was still capable of sustaining infection in feline monocytes up till 24hpi. However, the increase in infected monocytes and the resulting virus titre was less pronounced for FIPV-Δ3 than for wt-FIPV. These results showed that at least one of the ORF3 encoded accessory proteins should be intact for optimal replication. FIPV-Δ3 is distinguished from wt-FIPV 79-1146 by a deletion of only the 3a and 3b genes, since the 3c gene of wt-FIPV 79-1146 encodes for a truncated 3c protein (Volker, 2007), which also appears in 60-71.4% of *in vivo* FIPV strains (Chang et al., 2010; Pedersen et al., 2012). Thus, the somewhat lower replication capacity of FIPV-Δ3 in monocytes is most likely due to the lack of protein 3a and/or 3b. The use of the truncated wt-FIPV in present study can also explain the, at first sight, contrasting results obtained by Balint et al. (2012). They showed that an ORF3 truncation was needed for efficient FIPV type II replication in monocytes/macrophages. Our results demonstrate that ORF3c-truncated wt-FIPV 79-1146 indeed efficiently replicates in monocytes/macrophages, but that at least one of the other proteins (3a and 3b) should be intact to support FIPV replication in monocytes. Not surprisingly, infection with the double deletion mutant FIPV-Δ3Δ7 showed an additive effect of both ORF3 and ORF7 deletions. Consequently, FIPV-Δ3Δ7 reached lowest titres and lowest percentages of infected cells at 24hpi.

Finally, in this study it was observed that in fcwf cells FIPV-Δ7 and FIPV-Δ3Δ7 infection resulted in lower virus titres compared to wt-FIPV, whereas the titres reached by these viruses were not significant different in CrFK cells (data not shown). The significant lower titre obtained for FIPV-Δ7 and FIPV-Δ3Δ7 was explained by a lower production capacity of

these viruses in fcwf cells (10 times less infectious virus than wt-FIPV) compared to CrFK cells. This phenomenon could be ascribed to macrophage features of the fcwf cells (Jacobse-Geels and Horzinek, 1983). The impaired replication of FIPV-Δ7 and FIPV-Δ3Δ7 in fcwf cells, seen in this study, confirmed the role of ORF7 in efficient replication of FIPV in its host cells (monocytes/macrophages), suggesting a function in typical macrophage pathways.
In conclusion, it was found that deleting ORF7 from FIPV 79-1146 had a negative impact on the replication kinetic in feline monocytes. Thus, proteins encoded by ORF7 play a decisive role in sustaining the replication of FIPV in its *in vivo* target cell. The moderate effect of an ORF3 deletion from FIPV indicates that proteins encoded by ORF3 have only a supportive role in the replication. The results of this study will aid in the understanding of the role of accessory proteins in FCoV infections of monocytes and in the tropism switch of FIPV. Although it is still only a tip of the iceberg, our discoveries are a step forward in unravelling the function(s) of the accessory proteins of FCoVs and give indications for further research in the involvement of the accessory proteins (such as 7a and/or 7b) in antiviral pathways.

Acknowledgements

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Chapter 4

The ORF7-encoded Accessory Protein 7a of Feline Infectious Peritonitis Virus as a Counteragent against Interferon-alpha Induced Antiviral Response

Adapted from:

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Abstract

The type I interferon (IFN)-mediated immune response is the first line of antiviral defence. Coronaviruses, like many other viruses, have evolved mechanisms to evade this innate response, ensuring their survival. Several coronavirus accessory genes play a central role in these pathways but for feline coronaviruses this had never to our knowledge been studied. As it was demonstrated before that open reading frame (ORF) 7 is essential for efficient replication *in vitro* and virulence *in vivo* of feline infectious peritonitis virus (FIPV), the role of this ORF in the evasion of the IFN-α antiviral response was investigated. Deletion of ORF7 from FIPV 79-1146 rendered the virus more susceptible to IFN-α treatment. Given that ORF7 encodes two proteins 7a and 7b, it was further explored which of these proteins are active in this mechanism. Providing 7a protein in trans rescued the mutant FIPV-Δ7 from IFN sensitivity, which was not achieved by addition of 7b protein. Nevertheless, addition of protein 7a to FIPV-Δ3Δ7, a FIPV mutant deleted in both ORF3 and ORF7, could no longer increase the replication capacity of this mutant in the presence of IFN. These results indicate that FIPV 7a protein is a type I IFN antagonist and protects the virus from the antiviral state induced by IFN, but it needs the presence of ORF3 encoded proteins to exert its antagonistic function.

4.1 Introduction

Coronaviruses (CoVs) generally cause mild diseases during respiratory and intestinal infections in mammals and birds but may exceptionally lead to life-threatening diseases, such as severe acute respiratory syndrome (SARS) or Middle East respiratory syndrome (MERS) in humans and feline infectious peritonitis (FIP) in felids (Drosten et al., 2003; Ward et al., 1968; Zaki et al., 2012). Despite their economical and sentimental importance, information on the molecular mechanisms governing CoV virulence and pathogenesis is scarce. Feline infectious peritonitis virus (FIPV), which is the causal agent of the fatal systemic disease 'FIP' in cats, is one of the CoVs for which significant research efforts still have not resulted in a fully elucidated pathogenesis. FIPV share high genetic and structural similarity with feline enteric coronavirus (FECV) that causes at most a temperate, self-limiting diarrhoea as a result of a mild enteritis evoked by replication of the virus in enterocytes (Hayashi et al., 1982; Herrewegh et al., 1997; Kipar et al., 2010; Meli et al., 2004; Pedersen et al., 1981b). It has been shown that mutation from FECV to FIPV within an infected cat is the most probable means of acquiring FIP (Chang et al., 2010; Herrewegh et al., 1995; Licitra et al., 2013; Myrrha et al., 2011; Pedersen et al., 2012; Rottier et al., 2005; Vennema et al., 1998). The causal mutations will favour replication of the virus inside blood monocytes, causing a systemic spread of FIPV, resulting in an immune-mediated vasculitis and ultimately leading to death (Dewerchin et al., 2005; Rottier et al., 2005; Stoddart and Scott, 1989; Vennema et al., 1998). Although any cat that carries FECV is potentially at risk for developing FIP, it is typically seen that cats with an ineffective immune system are more likely to develop the disease (Addie et al., 1995).

Like all viruses, FIPV is forced to live as an intracellular parasite of its host cell. Viral replication and transcription occur in the cytoplasm, leading not only to new genome molecules and the typical nested set of subgenomic mRNAs, but also to dsRNA intermediates (Enjuanes et al., 2006; Gorbalenya et al., 2006; Spaan et al., 1988). Furthermore, FIPV does not encode the enzymatic machinery to translate its subgenomic mRNAs. Therefore, it is obliged to make use of the cellular apparatus for mRNA translation (Thompson and Sarnow, 2000). This dependency, together with the presence of viral dsRNA species, enables the invaded host cell to recognize the viral intruder and triggers diverse immune responses (Medzhitov and Janeway, 1997; Takeuchi and Akira, 2009). The secretion of type I interferon (IFNs), IFN-α and IFN-β, is the anchor of the innate host defence against virus infection. This drives both autocrine and paracrine signalling, which induces IFN-stimulated gene (ISG)

products with antiviral activities, resulting for instance in a global protein synthesis arrest. Because of the crucial importance in ensuring translation of mRNA, viruses have evolved strategies to breach these cellular defences (Gale and Sen, 2009; Grandvaux et al., 2002; Randall and Goodbourn, 2008; Taylor and Mossman, 2013). In general, CoVs express an impressive variety of viral proteins that act as modulators of the IFN gateway. Viral evasion proteins can degrade or inhibit IFN transcription factors or antagonize IFN signalling [nsp1 of SARS-CoV, protein 5a of mouse hepatitis virus (MHV)], as well as hijack cellular pathways or modulate ISG products [N protein of MHV, nsp2 of infectious bronchitis virus (IBV) and protein 7 of transmissible gastro-enteritis virus (TGEV)] (Cruz et al., 2011; Kamitani et al., 2006; Koetzner et al., 2010; Wang et al., 2009; Ye et al., 2007). For FIPV, not much is known about the battle between the body's defence mechanisms and the invading virus, but it seems likely that FIP only occurs if the virus has some means of circumventing the IFN response. In this respect, accessory proteins have been pointed out as of key importance for virus-host interactions. Given the impaired replication of FIPV deleted in the accessory ORF7 (Dedeurwaerder et al., 2013a) and the attenuation of these mutant viruses *in vivo* (Haijema et al., 2004), proteins encoded by this ORF deserve further attention. ORF7 encodes for two proteins. Protein 7a is a small hydrophobic protein of 101 AA (~10kDa) with a N-terminal cleavable signal sequence and a C-terminal transmembrane domain (Volker, 2007). It has a 72% sequence homology with protein 7 of TGEV (Cruz et al., 2011). Protein 7b is a soluble glycoprotein of ~24kDa (207AA), from which expression in natural infections has already been implied (Kennedy et al., 2008). It contains a KDEL-like endoplasmic reticulum (ER) retention signal (=KTEL) at its C-terminus (Vennema et al., 1992). This allows initial ER attachment, whereupon it is secreted from the infected cell.

In this study, it was investigated if the hampered replication seen for ORF7 deletion mutants of FIPV was explained by a higher susceptibility of these mutants for IFN-α and which proteins encoded by ORF7 were involved in this mechanism.

4.2 Material and methods

4.2.1 Viruses and cells

Crandell Rees feline kidney (CrFK) cells were used to obtain third passages of type II FIPV strain 79-1146. FIPV strain 79-1146 was kindly provided by Dr. P.J.M. Rottier (Faculty of Veterinary Medicine, Utrecht University, The Netherlands).

Felis catus whole fetus (fcwf-4) cells were used to obtain fifth passages of FIPV-Δ7, FIPV-Δ3 and FIPV-Δ3Δ7. This virus is a deletion mutant from type II FIPV strain 79-1146, kindly provided by Dr. Rottier (Faculty of Veterinary Medicine, Utrecht University, The Netherlands). It has been deleted in open reading frame (ORF) 7ab, using reverse genetics (Haijema et al., 2004).

293FT cell line, which is a fast-growing, highly transfectable clonal isolate derived from human embryonic kidney (HEK) cells transformed with the Simian virus 40 (SV40) large T antigen, were used for production of lentiviruses containing the 7a or 7b gene.

4.2.2 Generation of fcwf-4 cells stably expressing FIPV 7a and 7b protein.

The genes for FIPV 79-1146 7a and 7b proteins, with stop codons mutated into tyrosine and serine, respectively, were synthesized by Genscript (Piscataway, NJ, USA). Genes were cloned into a lentiviral vector pTRIP-CMV-GFP-WPRE. It was assured that the accessory 7a or 7b genes were in frame with the reporter green fluorescence protein (GFP) gene, eventually resulting in the expression of 7a and 7b proteins with GFP tag at their C-terminus.

To produce a lentivirus, three plasmids were transfected into a packaging cell line, 293FT, by the use of a calcium-phosphate transfection kit (Life Technologies). One plasmid, generally referred to as packaging plasmid, encodes the virion proteins, such as the capsid (gag gene) and the polymerase (pol gene). The second plasmid is the envelope vector coding the VSV-G protein. The third plasmid is the pTRIP-7a/7b-GFP transfer vector which contains the genetic material (7a/7b-GFP) to be delivered by the vector. After three days, lentiviral supernatant was harvested and was used to transduce fcwf-4 cells. 7a- or 7b-expressing fcwf-4 cells (fcwf-7a or fcwf-7b) were selected by fluorescence-activated cell sorting (FACS) using a FACSAriaIII cell sorter (BD Biosciences) on GFP expression and further cultivated. Expression and localisation of the 7a-/7b-GFP fusion proteins was verified by fluorescence microscopy (Leica Microsystems DMRBE) and Western blotting.

4.2.3 Antibodies for fluorescence staining

Primary antibody Calnexin Antibody C-20 (sc6465) was purchased with Cell signalling and used in 1/50 dilution. Primary monoclonal mouse antibody anti-Golgi 58K protein/ Formiminotransferase cyclodeaminase (FTCD) (G2404) was purchased with Sigma and used in 1/100 dilution. Secondary antibodies rabbit-anti-goat Alexa Fluor 594 was used in a 1/500 dilution and goat-anti-mouse Texas Red was used in a 1/100 dilution and both were purchased with Molecular Probes. Membrane staining was performed by incubation of the

cells with 2mM biotin (Thermo Scientific), followed by Streptavidin-Texas Red (Molecular Probes) staining (1/100). Cell nuclei were stained with Hoechst 33342 (Molecular Probes).

4.2.4 SDS-PAGE and western blotting

Stably transduced cells were scraped into medium on ice, washed in PBS buffer and lysed in lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 1% NP-40, 1mM NaF, 1mM Na_3VO_4, 1mM PMSF, protease inhibitor cocktail) for 1h at 4°C. Cell lysates were fractionated on a 12% polyacrylamide gel by SDS–PAGE and then transferred to a Hybond-P PVDF membrane. After blotting, the membranes were blocked in 5% non-fat dry milk in 0.1% PBS/Tween-20 overnight at 4°C. Following 1h incubation with primary ABfinity™ Recombinant Rabbit GFP Monoclonal antibody (Molecular Probes) and 1h incubation with secondary goat anti-rabbit HRP-conjugated antibody (DAKO cytomation), blots were developed by enhanced chemiluminescence (ECL Prime; GE Healthcare) and analyzed with ChemiDoc™ MP Imaging System (Bio-Rad). All incubation steps were done in blocking buffer.

4.2.5 IFN-α detection bioassay

Sera from 4 FIP (confirmed by post-mortem examination), 3 FECV negative cats and 3 FECV positive cats were used for the detection of *in vivo* IFN production. *In vitro* samples were taken by collecting supernatant of fcwf-4, fcwf-7a, fcwf-7b and fcwf-GFP cells infected with FIPV-wt or FIPV-Δ7 (multiplicity of infection (m.o.i.) of 0.02). One in two serial dilutions were made from the samples and virus in the samples was inactivated by UV treatment (CL-1000 Ultraviolet Crosslinker, UVP). IFN-α bioactivity was measured by a fcwf-4-cell-based bioassay. Fcwf-4 cells (3×10^4 cells/well in 100 µl) were incubated overnight at 37°C in two-fold dilutions of samples or recombinant feline IFN-α as the standard (two repeats per sample). Subsequently, vesicular stomatitis virus (VSV) was added to the wells, and the cells were incubated for 2 days. It is well known that VSV is extremely sensitive to the antiviral actions of IFN-α (Belkowski and Sen, 1987). Viability staining of the cells was done by incubation with 0.1% neutral red, and after washing the absorbance was read using a spectrophotometer (Repetto et al., 2008). The dilution mediating 50% protection was defined as 1 U of IFN-α/50µl.

4.2.6 Infection

Monolayers of cells (fcwf; fcwf-7a and fcwf-7b;) were treated with increasing amounts (0 to 10^3 U ml^{-1}) of recombinant feline IFN-α (R&D Systems) for 24 h prior to infection. Cells were then inoculated with FIPV-wt 79-1146, FIPV deletion mutants, FIPV-Δ7 and FIPV-Δ3Δ7 or VSV at a m.o.i. of 0.02. IFN-α remained present during infection. FIPV infected cells were fixed for staining and produced virus was harvested for virus titration at 18 hours post inoculation (hpi). Supernatant of VSV infected cells was harvested for virus titration at 18 hpi.

4.2.7 Detection of viral nucleocapsid protein expression in FIPV infected cells

Fcwf-4 cells were trypsinized and fixed with 4% paraformaldehyde. FIPV nucleocapsid expression was visualized with mouse IgG1 monoclonal antibody (mAb) 10A12 (produced and characterized in our laboratory) followed by fluorescein isothiocyanate (FITC)- or Alexa Fluor 647 (AF647)-labeled goat anti-mouse IgG secondary antibodies (Molecular Probes). Specificity of the antibody binding was assessed by substituting the primary anti-N antibody with a isotype-matched irrelevant antibody. The percentage of infected cells for every virus was determined using FACSDiva software on the FACSCanto flow cytometer (BD Biosciences) and percent reduction in N-positive cells in IFN-treated versus non-treated cells was calculated.

4.2.8 Virus titration

Total virus was obtained by scraping the cells in their supernatants. Cells and supernatant were transferred to an eppendorf and subjected to one freeze-thaw cycle. Virus titres were assessed by a 50% tissue culture infective dose assay using CrFK cells. The 50% end-point was calculated according to the method of Reed and Muench (Reed and Muench, 1938). Percent reduction in virus titre in IFN-treated versus non-treated cells was calculated.

4.2.9 Statistical analysis

Percent reduction in N-positive cells and virus titres in IFN-treated versus non-treated fcwf-4 cells (whether transduced with 7a-GFP, 7b-GFP, GFP or not) was calculated. Statistical analysis was performed by Mann-Whitney U test with SPSS 19.0 (SPSS Inc., Chicago, Illinois, USA). Significant differences were considered if $p \leq 0.05$.

4.3 Results

4.3.1 *In vitro* and *in vivo* IFN-α production during FIPV infection

Feline interferon alpha was produced in the supernatants of fcwf-4 cells infected with FIPV-wt and FIPV-Δ7, as detected by the IFN-α bioassay. No significant difference was observed between the wt virus and his mutant (Table 4.1A). In addition, it appears that protein 7a and 7b expression did not influence the IFN-α induction by infection as no significant differences could be found in comparison with infected GFP-expressing cells (Table 4.1B).

Table 4.1: IFN-α measurements in the supernatant of FIPV-wt and FIPV-Δ7 infected fcwf-4 cells (m.o.i. of 0.02) (A.) or fcwf-GFP, fcwf-7a and fcwf-7b cells (m.o.i. 0.02, 18hpi) (B.) , determined by an IFN bioassay. Every replicate was repeated twice and the average of the spectrophotometric absorbance was used to calculate the IFN-α titre. 'SD' represents the standard deviation of the three replicates. Differences were not statistically significant ($p>0.05$).

A. **Supernatant**	**IFN-α titre (U ml^{-1})**				
	Replication 1	**Replication 2**	**Replication 3**	**Mean**	**SD**
FIPV-wt 18hpi fcwf -4	100	63	122	**95**	**30**
FIPV-Δ7 18hpi fcwf -4	33	45	45	**48**	**16**
FIPV-wt 24hpi fcwf -4	870	698	504	**691**	**183**
FIPV-Δ7 24hpi fcwf-4	648	502	387	**512**	**131**

B. **Supernatant**	**IFN-α titre (U ml^{-1})**				
	Replication 1	**Replication 2**	**Replication 3**	**Mean**	**SD**
FIPV-wt fcwf-GFP	93	63	112	**89**	**25**
FIPV-wt fcwf-7a	100	122	145	**122**	**23**
FIPV-wt fcwf-7b	200	189	108	**166**	**50**
FIPV-Δ7 fcwf-GFP	82	69	70	**74**	**23**
FIPV-Δ7 fcwf-7a	91	37	100	**76**	**49**
FIPV-Δ7 fcwf-7b	91	60	120	**90**	**30**

Table 4.2. : IFN-α measurements in cat sera and FCoV diagnosis. Diagnosis of FECV positive cats was determined by IPMA (immunoperoxidase monolayer assay), detecting FCoV antibodies in the serum, and a real-time RT-PCR (optimized in our laboratory (Desmarets et al., 2013)) detecting FCoV RNA in the faeces. Yana was transiently infected at the moment of sample analysis and has now resolved the infection (no faecal shedding anymore). Chablis appears to be persistently infected with FECV as he is still shedding virus in his faeces (already followed up from October 2011). Baghera was a SPF cat that was orally inoculated with FECV UCD (Vermeulen, 2013). His faeces and sera were analysed at 5 days post inoculation (dpi) and 28dpi. No viral copies were detected in the faeces from 56dpi. FIP were verified in sick cats by post-mortem diagnosis and presence of FCoV nucleocapsid (N) expression in cells purified from ascites and in FIP lesions. N.A. = not analysed

Cat serum	**IFN-α titre (U ml^{-1})**	**FCoV antibody titre**	**FCoV RNA copies (g faeces)$^{-1}$**	**FIP symptoms and lesions**	**FCoV N expression in ascites cells or FIP granulomas**
FECV-negative cats					
10GK4	154	<40	N.A.	No	N.A.
Maloe	206	<40	N.A.	No	N.A.
Streepje	322	<40	N.A.	No	N.A.
FECV-infected cats					
Yana (transient)	722	3200	$10^{7.6}$	No	N.A.
Baghera 5dpi (transient – exp infection study)	2090	<40	$10^{10.7}$	No	N.A.
Baghera 28dpi (transient – exp infection study)	519	1600	$10^{8.0}$	No	N.A.
Chablis (persistent)	946	1600	$10^{6.8}$	No	N.A.
FIP cats					
10FK3	957	≥12800	N.A.	Ascites, granulomas on colon, caecum and lung lobe	Yes
10FK8	8571	≥12800	N.A.	Ascites, many granulomas on abdominal organs	Yes
10FK9	482	≥12800	N.A.	Ascites, no visible granulomas	Yes
12FK11	3686	≥12800	N.A.	Ascites, many granulomas on abdominal organs	Yes

In the sera of cats diagnosed with FIP, a significant higher amount of IFN-α was detected (482-8571U ml^{-1}) in comparison with the sera of conventional FECV negative healthy cats, in which up to 26 times less IFN-α (154-322U ml^{-1}) was found. Beside this, the IFN-α expression in the sera of FECV positive cats revealed also an elevated production level in comparison with the FECV negative cats. The sera of a cat (Baghera) experimentally infected with FECV UCD revealed that IFN-α levels were remarkably elevated in the early stage post inoculation (Table 4.2).

4.3.2 FIPV-wt is more resistant to IFN-α pre-treatment than its accessory ORF7 deletion mutant FIPV-Δ7

As shown in Figure 4.1, FIPV-wt was sensitive to IFN treatment, typically showing an approximately 10-fold inhibition of virus production and a reduction of N-protein positive cells of about 50% at the highest dose tested (1000 U ml^{-1}). Notwithstanding, both the amount of nucleocapsid expressing cells and virus titres were significantly more reduced for the FIPV-Δ7 after treatment with as little as 10 U ml^{-1} IFN-α. At the highest dose of fIFN-α, the drop of infectious titre was in the order of 100-fold and there was a reduction in N-protein positive cells of 75%.

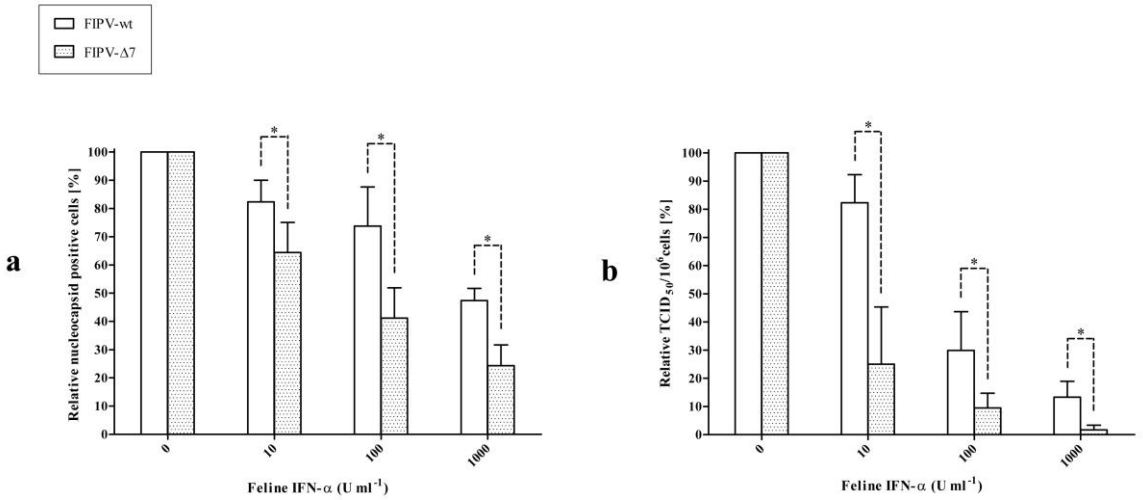

Figure 4.1: Effects of fIFN-α on replication of FIPV-wt and FIPV-Δ7 in fcwf-4 cells. Monolayers of fcwf-4 cells were untreated or pre-treated with different concentrations of fIFN-α (0-1000 U ml-1) for 24h. The cells were then infected with FIPV-wt and FIPV-Δ7. 18h post inoculation, cells were collected and fixed for immunofluorescence staining for the detection of viral nucleocapsid-expressing cells with flow cytometry (a). On the other hand, cells were also harvested and the titre of each virus was determined on CrFK cells (b). The efficacy of fIFN-α treatment was measured by the ratio of nucleocapsid positive cells or the titre of untreated cells to nucleocapsid positive cells or the titre of IFN-treated cells. The data represent means ± standard deviation (SD) of four replicates. P-values were calculated by the Mann-Whitney U test. Significant difference is indicated with * ($p \leq 0.05$).

4.3.3 Protein 7a and 7b localization in fcwf-4 cells

In assessing whether the absence of 7a and/or 7b protein during FIPV infection was responsible for the higher susceptibility for fIFN-α, fcwf-4 cells stably expressing FIPV 7a or 7b protein were generated. In order to detect 7a/7b protein, a GFP tag was inserted at the C-terminus of 7a/7b protein. Fcwf-4 stably expressing the GFP tag alone were used as control cells for the experiments. Expression was confirmed by western blot and immunofluorescence. The protein bands detected by western blot analysis proved that the accessory proteins were fused to GFP (Figure 4.2). We detected the expected shift in protein size with reference to GFP alone, knowing that the molecular weight of 7a protein is around 10kDa and of 7b protein is around 24kDa (Volker, 2007).

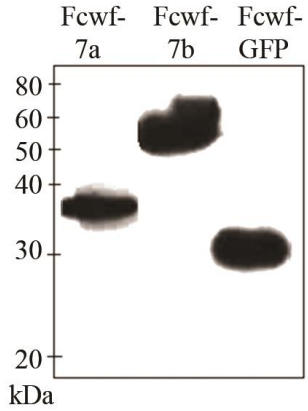

Figure 4.2: Western blot analysis of lysates of fcwf-4 cells transduced with 7a-GFP, 7b-GFP or GFP alone. Proteins are detected with ABfinity rabbit GFP monoclonal antibody (Life Technologies).

As shown by fluorescence microscopy, 7a-GFP protein is present in the cytoplasm, co-localizing mainly with ER and Golgi apparatus. 7b-GFP is targeted to the perinuclear region (Golgi apparatus), from where it disperses in the cytoplasm as dense spots, to eventually reach the cell membrane (Figure 4.3). In contrast to the 7a- and 7b-GFP fusion proteins, GFP alone is present in all cell compartments. This means that the accessory proteins are determining the localization of their GFP-fusion proteins.

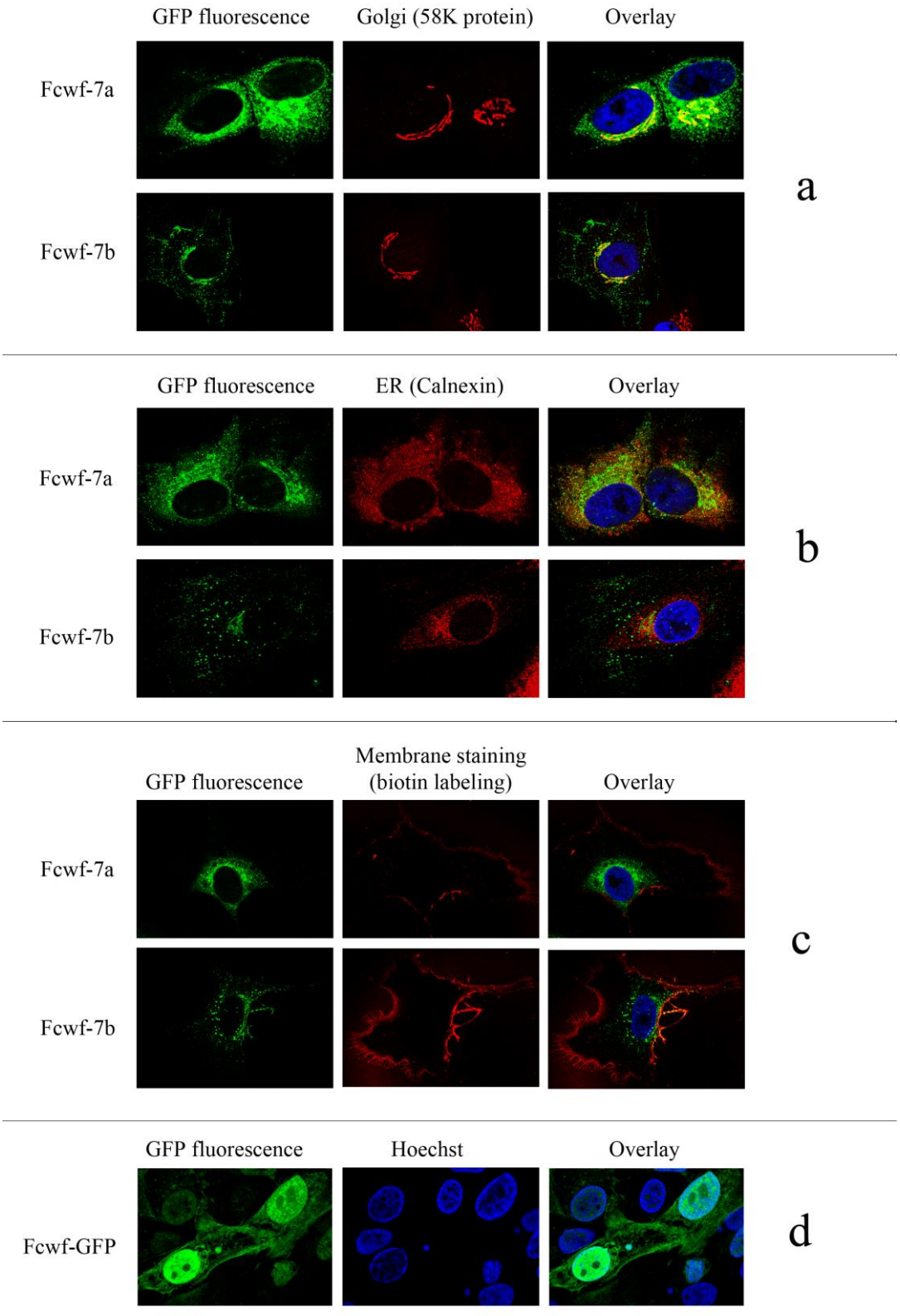

Figure 4.3: Confocal microscopic images of fcwf-4 cells transduced with 7a-GFP or 7b-GFP and stained with (a): 58K protein (Golgi complex marker); (b): calnexin (ER marker) or (c): biotin (membrane marker). (d): Representation of fcwf-4 cells transduced with GFP alone, which are used as control cells.

4.3.4 Protein 7a but not 7b restored the inhibitory effect of fIFN-α on FIPV-Δ7 replication

The effect of 7a or 7b protein provided in trans to FIPV-Δ7 on fIFN-α susceptibility was analyzed. FIPV-Δ7 infection of fcwf-4 cells expressing GFP alone, were used as control cells, excluding the effect of GFP on infection.

As previously observed with the untransduced fcwf-4 cells, there was a significant reduction of FIPV-Δ7 replication in the fcwf-GFP cells after fIFN-α treatment (80% reduction in N-protein positive cells and 200-fold drop of infectious titre). Under this fIFN-α pressure, 7a-GFP protein, but not 7b-GFP protein, has the capacity to significantly elevate the nucleocapsid expression and virus production of FIPV-Δ7 in comparison with GFP alone (Figure 4.4). P-values were represented in Table 4.2. In the presence of protein 7a, the reduction in viral N-producing cells and virus production was only 50% and 14-fold at the highest dose of IFN-α, respectively, which was comparable to the IFN-sensitivity of FIPV-wt. At low IFN-α levels, 7b-GFP protein had some positive effect on the amount of cells expressing viral N-protein, but this could not reach a significant level (p=0.1714). Providing 7a-GFP or 7b-GFP protein in trans to FIPV-wt induced no significant increase in titre or viral nucleocapsid protein expressing cells under IFN pressure (p>0.3).

Table 4.2: P-values determined by Mann-Whitney U test on data of FIPV-Δ7 in fcwf-7a/7b versus fcwf-GFP cells, as represented in Figure 4.4. Significant differences are indicated by * ($p \leq 0.05$)

Interferon concentration	Fcwf-7a versus fcwf-GFP infected with FIPV-Δ7		Fcwf-7b versus fcwf-GFP infected with FIPV-Δ7	
	P-values N expression	P-values $TCID_{50}$	P-values N expression	P-values $TCID_{50}$
10U ml^{-1} IFN	0.0286*	0.0286*	0.1714	0.3429
100U ml^{-1} IFN	0.0286*	0.0286*	0.1714	0.4429
1000U ml^{-1} IFN	0.0286*	0.0286*	0.3429	0.3858

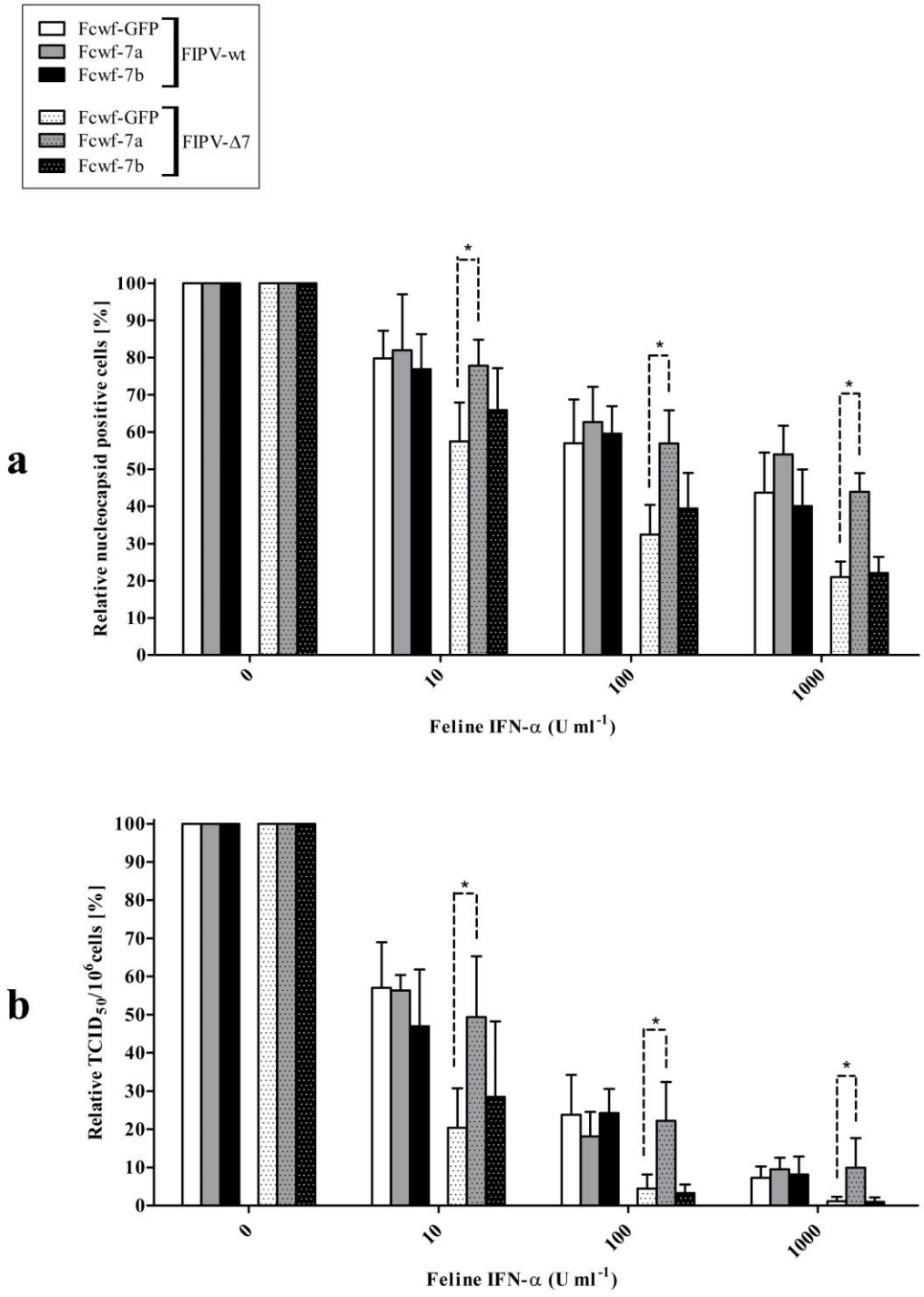

Figure 4.4: Effects of fIFN-α on replication of FIPV-Δ7 in fcwf-4 cells expressing either GFP alone, 7a-GFP or 7b-GFP. Experiments and analysis were performed like described in the legend of Figure 4.1. Nucleocapsid positive cells were shown in (a). Titration data was shown in (b). The data represent means ± standard deviation (SD) of minimum three replicates. *P*-values were calculated by the Mann-Whitney U test. Significant difference is indicated with * ($p \leq 0.05$).

4.3.5 Protein 7a does not affect VSV sensitivity towards IFN-α

Previous results emphasize the important role of protein 7a during IFN-α antagonism, and it was our purpose to further evaluate its mechanism. Therefore, it was examined if the protein could function on its own or if it needs the expression of other FCoV encoded proteins. To investigate this, protein 7a was provided in trans to a highly IFN-sensitive VSV virus and the effect on replication under IFN-α pressure was analysed. As shown by viral $TCID_{50}$, VSV replication was hampered to the same amount in the control cells as in the cells expressing protein 7a at all IFN concentrations tested (p=0.35 at 10U IFN-α and p=0.5 at 100U and 1000U IFN-α) (Figure 4.5). So, expression of protein 7a does not detectably affect the IFN-sensitivity of VSV. It should be mentioned that VSV was far more sensitive to IFN treatment than FIPV-wt, with more than 100 times reduction of viral titre at the lowest IFN dose (10U) tested and 1000 times less viral growth in the presence of 1000U IFN-α.

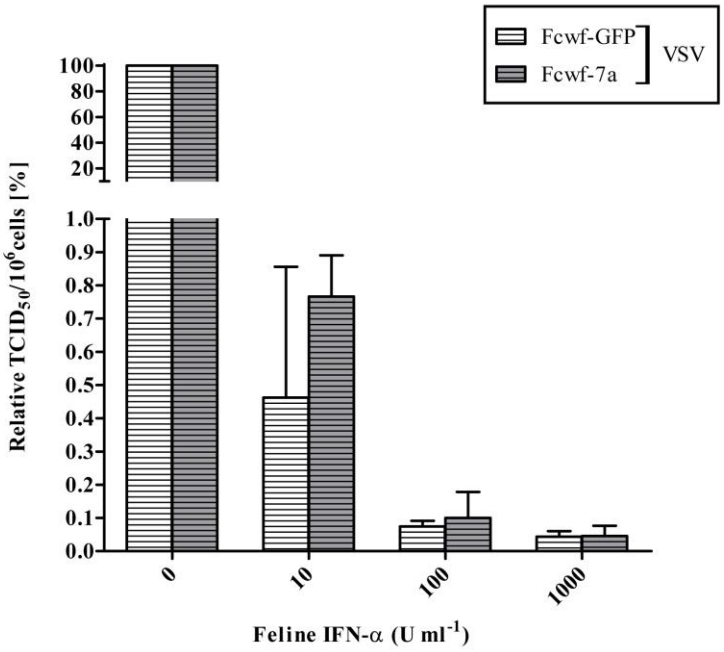

Figure 4.5: Effects of fIFN-α on VSV growth curves in fcwf-4 cells expressing either GFP alone or 7a-GFP. Experiments and analysis were performed like described in the legend of Figure 4.1a. The data represent means ± standard deviation (SD) of three replicates. P-values were calculated by the Mann-Whitney U test. Significant difference would have been indicated with * ($p \leq 0.05$).

4.3.6 FIPV-Δ3 and FIPV-Δ3Δ7 replication was less efficient than that of FIPV-wt after IFN-α treatment

Given the fact that protein 7a does not affect IFN-sensitivity of VSV, protein 7a might need the expression of other FCoV encoded protein to exert its anti-IFN function. As also 2 other mutants of FIPV, one deleted in ORF3 (FIPV-Δ3) and one deleted in both accessory ORFs (FIPV-Δ3Δ7) were attenuated *in vivo* and showed a reduced replication efficiency in feline monocytes *in vitro* (Dedeurwaerder et al., 2013a; Haijema et al., 2004), it was interesting to investigate their sensitivity to IFN-α. Figure 4.6 demonstrates that these two accessory protein mutants of FIPV encountered a similar negative effect on their replication after IFN-α treatment as was demonstrated for FIPV-Δ7 ($p=0.05$ in comparison with FIPV-wt, both N expression as $TCID_{50}$ results, at all IFN concentrations tested).

4.3.7 Protein 7a does not affect IFN-sensitivity of FIPV-Δ3Δ7

The results described above, specifically that (i) protein 7a cannot reverse the IFN-sensitivity of VSV and (ii) FIPV-Δ3 shows a similar sensitivity to IFN treatment as FIPV-Δ7, indicate that 7a is not able to completely antagonize the IFN induced antiviral response without the presence of ORF3. This could be further evaluated by investigating the effect of providing 7a in trans to FIPV-Δ3Δ7. As shown in Figure 4.7, the presence of 7a does not significantly increase the replication efficiency of FIPV-Δ3Δ7 in comparison with GFP ($p\geq0.2$). The only exception on this, was that protein 7a did induce a significant ($p=0.05$) increase in viral titre in the presence of the highest IFN dose, but still it could not reach similar levels as FIPV-wt could under this IFN pressure. In general, protein 7a could no longer restore the replication efficiency of FIPV-Δ3Δ7 to the phenotype of FIPV-wt as it could for FIPV-Δ7, supporting a regulatory collaboration between 7a protein and ORF3 encoded protein(s) to target the IFN-induced antiviral response.

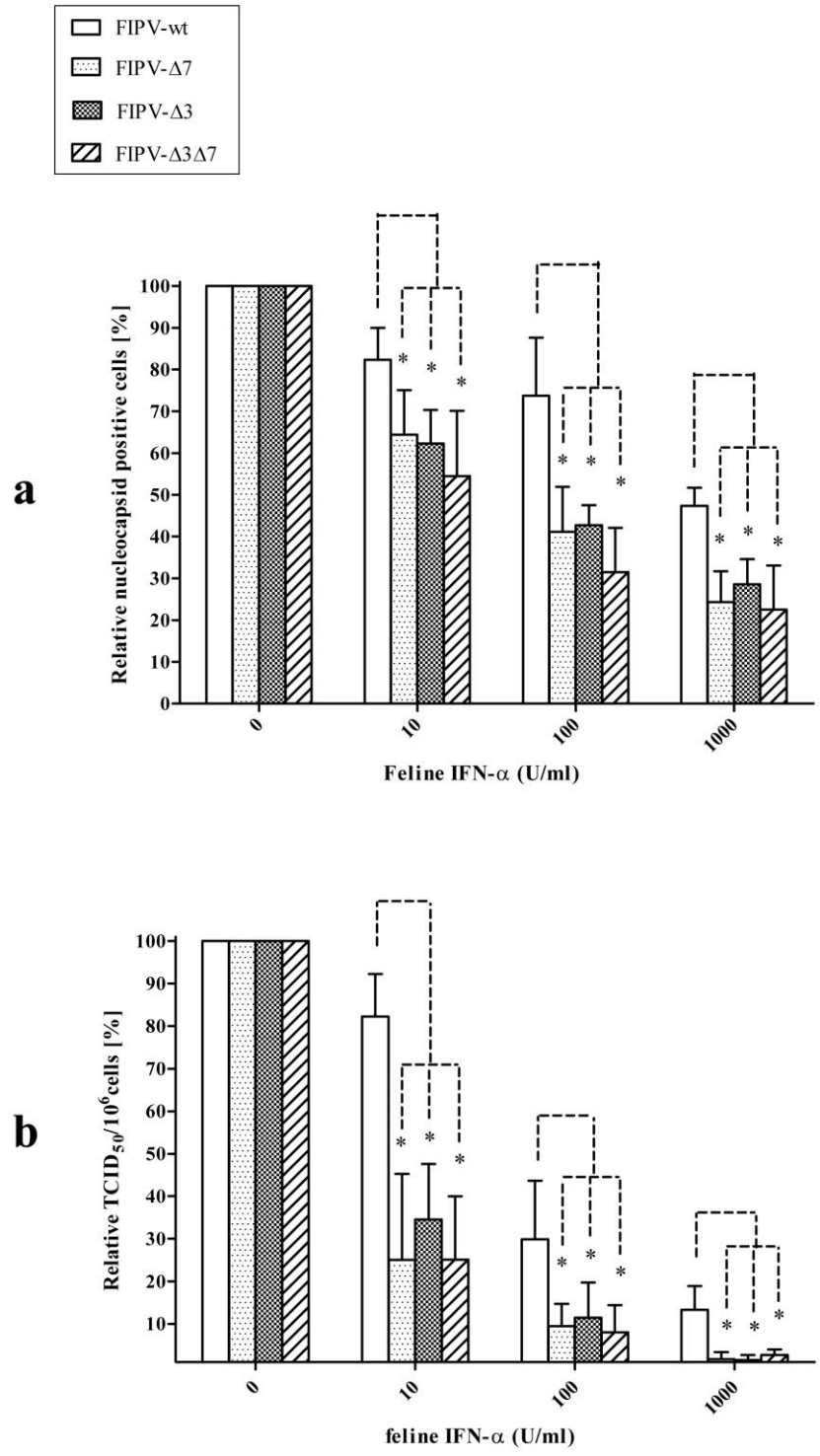

Figure 4.6: Effects of fIFN-α on replication of FIPV-wt, FIPV-Δ7, FIPV-Δ3 and FIPV-Δ3Δ7 in fcwf-4 cells. This figure represents Figure 4.1 extended with the replication results of FIPV-Δ3 and FIPV-Δ3Δ7 under IFN pressure. Experiments and analysis were performed like described in the legend of Figure 4.1. Nucleocapsid positive cells were shown in (a). Titration data was shown in (b). The data represent means ± standard deviation (SD) of minimum three replicates. P-values were calculated by the Mann-Whitney U test. Significant difference is indicated with * ($p \leq 0.05$).

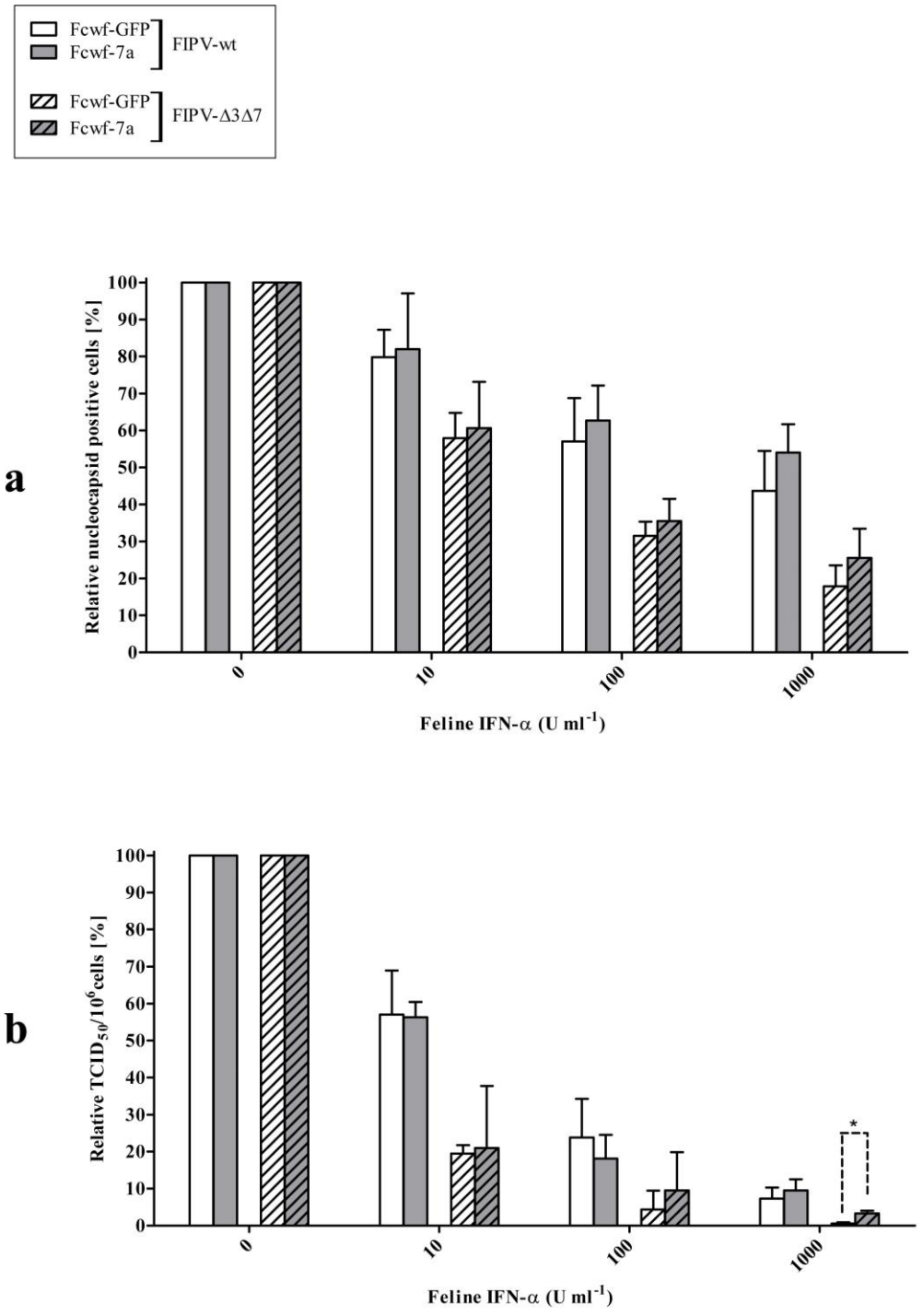

Figure 4.7: Effects of fIFN-α on replication of FIPV-Δ3Δ7 in fcwf-4 cells expressing either GFP alone or 7a-GFP. Experiments and analysis were performed like described in the legend of Figure 4.1. Data points of FIPV-wt were identical as those in Figure 4.4. Nucleocapsid positive cells were shown in (a). Titration data was shown in (b).The data represent means ± standard deviation (SD) of minimum three replicates. P-values were calculated by the Mann-Whitney U test. Significant difference is indicated with * ($p \leq 0.05$).

4.4 Discussion

Circumventing the early innate immune response, characterized by IFN induction, is a critical step for viruses to establish *in vivo* infections. All classes of viruses appear to encode proteins that act as modulators of one or more steps of the IFN signalling pathways (Gale and Sen, 2009). Among viruses, RNA viruses typically are the most potent inducers of IFN, consequently leading to the discovery of an impressive array of mechanisms used by these viruses to surmount the IFN-induced antiviral pathway. The last decade, some of these viral IFN-antagonizers, often accessory proteins, have also been identified in coronaviruses. These proteins are either involved in the inhibition of IFN synthesis (such as ORF3b, ORF6 and N protein of SARS-CoV (Kopecky-Bromberg et al., 2007)) or are circumventing the IFN signalling pathway (such as ORF7 protein of TGEV, ORF5a and N proteins of MHV and ORF3b, ORF6 and 7a protein of SARS-CoV) (Cruz et al., 2011; Koetzner et al., 2010; Kopecky-Bromberg et al., 2006; Ye et al., 2007). To date, there has been no description of an IFN-evader for feline coronaviruses. Nevertheless, research results from the last decades suggest that FCoVs may also counteract the IFN-induced antiviral pathway. It has been suggested many times before that ORF7 could play a role during virus-host interactions, but no specific function has been designated to one of the proteins encoded by this ORF before (Kennedy et al., 2001; Kennedy et al., 2008; Lin et al., 2009). Both impaired replication of FIPV deleted in the accessory ORF7 (Dedeurwaerder et al., 2013a), and attenuation of this mutant virus *in vivo* (Haijema et al., 2004), as well as the close genetic similarity with ORF7 protein of TGEV (Cruz et al., 2011) led to the hypothesis that proteins encoded by FIPV ORF7 may be involved in an IFN evasion mechanism.

We demonstrated that FIPV infection induced IFN-α, both *in vitro* and *in vivo*. The *in vitro* fIFN-α sensitivity assays showed that FIPV countermeasures are not absolute as FIPV-wt was to some extent sensitive to IFN-α pre-treatment. This supports the typical chronic interplay between FCoV and its host, as replication *in vivo* is mostly restricted to a low percentage of cells and the virus can persist for a couple of months without inducing any symptoms. Complete IFN activity disruption would also not be beneficial for the virus, as cells could interpret this as a stress signal, activating cellular apoptosis and destroying the cell before virus assembly has occurred. Notwithstanding the significant amount of IFN production *in vivo* and *in vitro*, it cannot restrict the virus replication sufficiently to prevent a fatal disease outcome. This can also explain why IFN-α administration to FIP cats is not sufficient as a treatment (Weiss et al., 1990). This observation indicates the presence of FIPV

encoded proteins (like 7a and/or 7b, for example) that block IFN actions downstream the IFN synthesis.

In this article, we have shown that IFN-α markedly impaired the replication of FIPV with a deleted ORF7 (FIPV-Δ7) compared to FIPV-wt. In addition, this can be fully complemented by providing 7a accessory protein of the FIPV-wt in trans, suggesting that the 7a accessory protein functions as an agent that interferes with the innate IFN-α mediated immune response. The presence of protein 7a in advance to infection could not reduce the IFN-α production by FIPV which indicates that 7a protein antagonizes the downstream IFN-production cascade. Further evaluating its mechanism, elucidated that protein 7a could only interfere with the IFN-α antiviral response in the presence of one or more proteins encoded by ORF3 (3a or 3b). This conclusion was made from the fact that (i) protein 7a could no longer restore the IFN-α sensitivity of FIPV-Δ3Δ7 or VSV and (ii) all accessory ORF mutants of FIPV experienced a similar negative effect of IFN-α treatment. Most likely, the collaboration of protein 7a and ORF3 encoded proteins eventually allows efficient replication of FIPV-wt. Whether this cooperation is characterized by a direct interaction of these proteins has to be further investigated. On the other hand, it is also possible that they function on different IFN induced pathways but which result in the same antiviral effect, being, for instance, inhibition of protein synthesis (Schoggins et al. 2011). Blocking both of these IFN induced pathways could be essential for the virus to overcome the overall negative effect of IFN.

Deleting accessory ORFs from FIPV-wt did not render this mutant fully susceptible to IFN, revealing that other viral proteins (nsp and/or structural proteins) can also participate in the IFN antagonism.

The fact that 7b protein is not able to restore virus infectivity under IFN-α pressure, indicates that this accessory protein is fulfilling another function. The expression pattern of 7b could represent the slow export of the protein by microsomes, as suggested by Vennema et al. (Vennema et al., 1992), implying a role in the extracellular environment of infected target cells. Given the sequence distribution of ORF7, it is not that surprising that 7b protein is playing a minor role compared to protein 7a in defence against first-line antiviral actions of infected cells. As 7b is translated from ORF7 by ribosomal leaky scanning, it is very likely that protein expression levels are low in comparison to 7a. Moreover, the sequence of 7a gene is far more conserved between patho- (FIPV and FECV) and serotypes (type I and II), compared to that of the 7b gene, which may be in line with an important role for the 7a protein.

This IFN-α evasion can be added to the list with earlier described immune-evasion strategies of FIPV, like retention, internalization, complement blockage and suppression of lymphocyte proliferation (Cornelissen et al., 2007, 2009; Dewerchin et al., 2008; Vermeulen et al., 2013).
It has been demonstrated before that FIPV-Δ7 replication is impaired in peripheral blood monocytes, as well as in fcwf-4 cells, which was not seen in Crandell Rees Feline Kidney (CrFK) cell line (Dedeurwaerder et al., 2013). Performing the IFN-α bioassay on fcwf-4 and CrFK cells revealed that VSV replication in CrFK cells was far less sensitive to fIFN-α pre-treatment than on fcwf-4 cells (data not shown). These observations may indicate that CrFK cells may possess less IFN-receptors than fcwf-4 cells and therefore be less sensitive against antiviral activities, allowing higher replication of IFN-sensitive viruses, which was previously suggested by Mochizuki *et al.* (Mochizuki et al., 1994).
In summary, we demonstrated that protein 7a may be a key player in the circumvention of the antiviral defence of the host. Moreover, a cooperation between protein 7a and ORF3 encoded proteins appears to be essential to completely abolish IFN-α mediated restriction of viral replication. Although the exact mechanism of counteraction remains unclear, this leaves abundant scope for further unravelling the molecular mechanism employed by protein 7a to antagonize the IFN-induced antiviral response. This may aid to define innovative antiviral reagents that work by preventing FIPV from blocking specific cellular activities, supporting the defeat of this awful disease.

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Chapter 5

Protein 7a of Feline Infectious Peritonitis Virus Interferes with Phosphorylation of Eukaryotic Translation Initiation Factor Alpha

Manuscript in preparation:

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Abstract

The feline infectious peritonitis virus (FIPV) genome contains two accessory open reading frames (ORF3 and ORF7) that encode five accessory genes: 3a, 3b, 3c, 7a and 7b, and that are of substantial importance for virus virulence in the host. Nevertheless, their exact functions have not yet been elucidated. Recently, we discovered a role for protein 7a in counteracting the interferon alpha (IFN-α)-induced antiviral response. Protein 7a is only present in members of coronavirus genus α1, and its closely related transmissible gastro-enteritis virus (TGEV) counterpart, protein 7, has previously been identified as an inhibitor of phosphorylation of the eukaryotic translation initiation factor eIF2α by interacting with the cellular protein phosphatase 1 (PP1). Here, the involvement of FIPV protein 7a in this pathway was studied. The results showed that ectopically expressed protein 7a potently reduces eIF2α phosphorylation. Mutation of the predicted PP1 binding motif in protein 7a impaired interference with phosphorylation of eIF2α, suggesting that PP1 is involved in this process. In addition, infection studies with accessory ORF deletion mutants of FIPV suggested a redundant antagonizing role for both ORF3 and ORF7 genes on the eIF2α phosphorylation pathway, which opens new lines for further research. In summary, the accessory protein 7a of FIPV has the previously uncharacterized ability to suppress phosphorylation levels of eukaryotic translation initiation factor eIF2α, most probably via protein phosphatase PP1.

5.1 Introduction

RNA viruses are among the most potent type I interferon (IFN) inducers. IFN-α/β are key components of the innate immune system and bind to their specific receptor, triggering a signalling cascade that culminates in the transcription of a large number of IFN-stimulated genes (ISGs) that encode antiviral proteins. Consequently, the cell is protected from virus infection until the antiviral proteins are degraded, a process that takes several days. The antiviral state in IFN-treated cells results from the synthesis of multiple enzymes, including two important enzymes that lead to a global arrest of protein synthesis. One critical antiviral IFN-induced enzyme is dsRNA-dependent protein kinase (PKR), which, upon activation by viral dsRNA, triggers the phosphorylation of the eukaryotic translation-initiation factor 2α (eIF2α) at serine 51. This results in the interruption of viral and cellular protein elongation, effectively dampening virus replication and production. PKR is present in most cells at basal levels even in the absence of IFN. IFN induces the synthesis of PKR but viral dsRNA is necessary to mediate PKR dimerization and activation (Clemens, 1997; Clemens and Elia, 1997; Proud, 1995).

The effectiveness of the IFN response has driven many viruses to develop specific mechanisms that antagonize the production or actions of IFNs. Viral countermeasures against the IFN response are rarely absolute, but the speed and efficiency by which a given virus circumvents the IFN response, may be a critical determinant for host range and pathogenicity (Goodbourn et al., 2000). In general, most, if not all, classes of viruses appear to express an impressive variety of viral proteins that counteract the PKR function. Some of them restrain PKR activity by direct binding to PKR itself or to dsRNA [US11 of herpes simplex virus 1 (HSV-1), NS5A and E2 of hepatitis C virus (HCV)] (Cassady et al., 1998; Gale et al., 1997; Taylor et al., 1999), while others inhibit phosphorylation of eIF2α by indirect mechanisms such as the modulation of host protein phosphatase 1 (PP1), a serine/threonine (Ser/Thr) phosphatase that is able to directly dephosphorylate the translation factor eIF2α, thereby restoring its activity. PP1 participates in numerous cellular functions by interacting with its PP1 catalytic subunit (PP1c) with several regulatory partners. These binding partners determine the substrate specificity, cellular localisation and activity of PP1 (Aggen et al., 2000; Cohen, 2002). Viruses can carry proteins that mimic cellular proteins (e.g. GADD34) (Brush et al., 2003) that interact with PP1 and recruit it to the eIF2α phosphorylation pathway [e.g. protein 7 of transmissible gastro-enteritis virus (TGEV), IE180 of porcine respiratory

coronavirus (PRV), γ34.5 of HSV-1] (Cruz et al., 2011; Li et al., 2011b; Van Opdenbosch et al., 2012).

Feline infectious peritonitis virus (FIPV) is an RNA virus, belonging to the family of the *Coronaviridae*, that causes lethal systemic infection of felids. Like for all coronaviruses, both viral and cellular proteins determine virus replication and their interplay may explain the virus pathogenesis (Enjuanes et al., 2006). Recently, we found that replication of a FIPV strain carrying a deletion in ORF7 (FIPV-Δ7) was significantly more hampered after fIFN-α treatment than the wt-virus. Providing protein 7a in trans reversed this phenotype (Dedeurwaerder et al., 2014). Protein 7a is one of the accessory proteins of FIPV 79-1146 and is expressed from ORF7. It is a small hydrophobic protein of 101 AA (~10kDa) with a N-terminal cleavable signal sequence and a C-terminal transmembrane domain (Volker, 2007). Protein 7a shows a 72% sequence homology with TGEV protein 7, including an important PP1 binding motif. As described above, TGEV protein 7 has been shown to interfere with the IFN-induced antiviral response through inhibition of eIF2α phosphorylation via recruitment of PP1 (Cruz et al., 2011). Based on this knowledge, the aim of the current study was to determine whether protein 7a of FIPV prevents phosphorylation of eIF2α.

5.2 Materials and methods

5.2.1 Viruses and cells

Crandell Rees feline kidney (CrFK) cells were used to obtain third and fifth passages of type II FIPV strain 79-1146 and its deletion mutants FIPV-Δacc (collective term for FIPV-Δ3, FIPV-Δ7 and FIPV-Δ3Δ7), respectively. The viruses were kindly provided by Dr. Rottier (Faculty of Veterinary Medicine, Utrecht University, The Netherlands). FIPV-Δacc have been deleted in open reading frame (ORF) 3abc (FIPV-Δ3), 7ab (FIPV-Δ7), or both (FIPV-Δ3Δ7), using reverse genetics (Haijema et al., 2004).

293FT cell line, a fast-growing, highly transfectable clonal isolate derived from human embryonic kidney (HEK) cells transformed with the SV40 large T antigen, was used for production of lentiviruses.

Felis catus whole foetus (fcwf) cells were used to evaluate the eIF2α phosphorylation induction after transduction, ER stressor treatments and infections.

5.2.2 Generation of fcwf cells stably expressing GFP or FIPV protein 7a or 7aPP1cDEL

Fcwf cells stably expressing GFP, FIPV protein 7a-GFP or 7aPP1cDEL-GFP alone were generated as previously described (Dedeurwaerder et al., 2014). Briefly, the gene encoding type II FIPV 79-1146 protein 7a or 7aPP1cDEL was cloned by Genscript into a lentiviral vector pTRIP-GFP, creating pTRIP-7a-GFP or pTRIP-7aPP1cDEL-GFP, respectively. The pTRIP-7aPP1cDEL-GFP plasmid encodes FIPV protein 7a with a deletion comprising amino acids 82 to 85, which represents the predicted PP1c binding motif (RVIF). It was assured that the accessory gene was in frame with the green fluorescence protein (GFP) reporter gene, resulting in the expression of 7a or 7aPP1cDEL protein with a GFP tag at its C-terminus. Lentiviruses containing either pTRIP-GFP, pTRIP-7a-GFP or pTRIP-7aPP1cDEL-GFP were produced in 293FT cells and were used to transduce fcwf cells. 7a-GFP, 7aPP1cDEL-GFP or GFP-expressing fcwf (fcwf-7a, fcwf-7aPP1cDEL or fcwf-GFP) were selected by fluorescence-activated cell sorting (FACS) using a FACSAriaIII cell sorter (BD Biosciences) on GFP expression and further cultivated.

5.2.3 Induction of eIF2α phosphorylation by a chemical ER stressor or infection

To induce ER stress and subsequent phosphorylation of eIF2α, cells (fcwf, fcwf-7a, fcwf-7aPP1cDEL and fcwf-GFP) were pre-treated for 1h with 1µM thapsigargin (TG) (Invitrogen). Thapsigargin, an inhibitor of sarcoplasmic/ER Ca^{2+}ATPases, induces eIF2α phosphorylation by activating the pancreatic endoplasmic reticulum PERK protein kinase (Wong et al., 1993).

The infection studies were performed by inoculating monolayers of cells (fcwf, fcwf-7a and fcwf-7aPP1cDEL) with FIPV-wt 79-1146 or FIPV-Δacc, at a multiplicity of infection (m.o.i.) of 0.02 for 18h.

Cells were lysed for immunoblotting in lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 1% NP-40, 1mM NaF, 1mM Na_3VO_4, 1mM PMSF, protease inhibitor cocktail) for 1h at 4°C.

5.2.4 SDS-PAGE and Western blotting

Cell lysates were fractionated on a 12% polyacrylamide gel by SDS–PAGE and then transferred to a Hybond-P PVDF membrane. After blotting, the membranes were blocked in 5% non-fat dry milk in 0.1% PBS/Tween-20 (PBS-T) for 1h at room temperature or overnight at 4°C. The blots were further incubated with primary antibodies (overnight at 4°C

for mouse anti-eIF2α (L57A5) (Cell Signalling) and rabbit anti-phospho-eIF2α (D9G8) (Cell Signalling) or 1h at room temperature for mouse IgG1 anti-N (10A12) (produced and characterized in our laboratory) and ABfinity™ Recombinant Rabbit GFP Monoclonal antibody (Molecular Probes)). After washing for three times with 0.1% TBS/Tween-20 (TBS-T) or PBS-T, the blots were incubated for 1h at room temperature with HRP-conjugated secondary antibodies (DAKO cytomation) or HRP-labeled Anti-alpha Tubulin [ab40742] (Abcam). After three washing steps, blots were developed by enhanced chemiluminescence (ECL Prime; GE Healthcare) and analyzed with ChemiDoc™ MP Imaging System (Bio-Rad). Protein band intensities were measured using Image J software. Phospho-eIF2α/total eIF2α ratios of treated samples were determined and normalized against phospho-eIF2α/total eIF2α ratios of mock samples.

5.2.5 Statistical analysis

Statistical analysis was performed using GraphPad Prism software using a Mann-Whitney U-test. Differences were considered significant when $p \leq 0.05$.

5.3 Results

5.3.1 Expression of accessory protein 7a interferes with eIF2α phosphorylation

Phosphorylation of eIF2α was induced in fcwf cells by treatment with thapsigargin. Figure 5.1 shows that this treatment effectively stimulated phosphorylation of eIF2α (p=0.05).

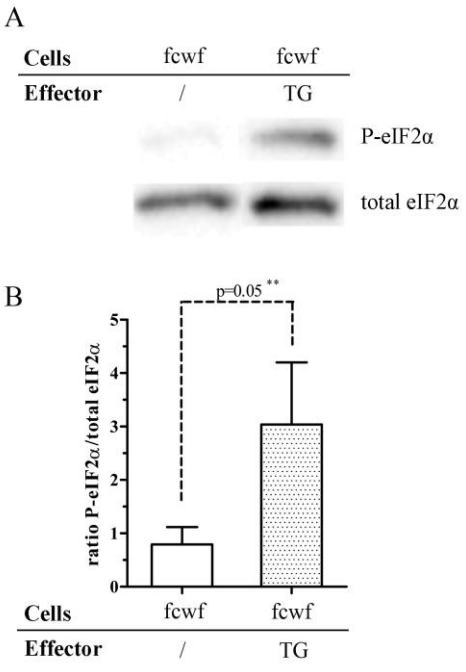

Figure 5.1: Thapsigargin (TG) treatment causes eIF2α phosphorylation of fcwf cells. (A) Western blot detection for total and phosphorylated eIF2α. (B) Graphical representations of P-eIF2α/total eIF2α ratios of three independent replicates. Asterisks indicate statistically significant differences ($p \leq 0.05$).

To elucidate whether protein 7a of FIPV 79-1146 is capable of antagonizing this phosphorylation, relative phospho-eIF2α/total eIF2α ratios upon thapsigargin addition were compared between fcwf cells stably expressing the 7a-GFP fusion protein and the control fcwf cells expressing GFP alone. Western blot analysis confirmed the expression of the 7a-GFP fusion protein and the GFP control protein (Figure 5.2A, lanes 2-3). First of all, transduction of recombinant GFP proteins in fcwf cells (fcwf-GFP cells) clearly induced higher eIF2α phosphorylation compared to non-transduced fcwf cells (Figure 5.2A, lanes 1-2). This may indicate that the transduced fcwf cells are more prone to eIF2α phosphorylation induction due to stress than untransduced cells, which makes the fcwf-GFP cells better controls for the functional study of protein 7a-GFP than the untransduced fcwf cells. Coupling 7a to GFP, the phosphorylation induction was significantly diminished in comparison to the GFP controls (p=0.0317), indicating that the expression of protein 7a interferes with eIF2α (Figure 5.2A, lanes 2-3, Figure 5.2B, columns 2-3).

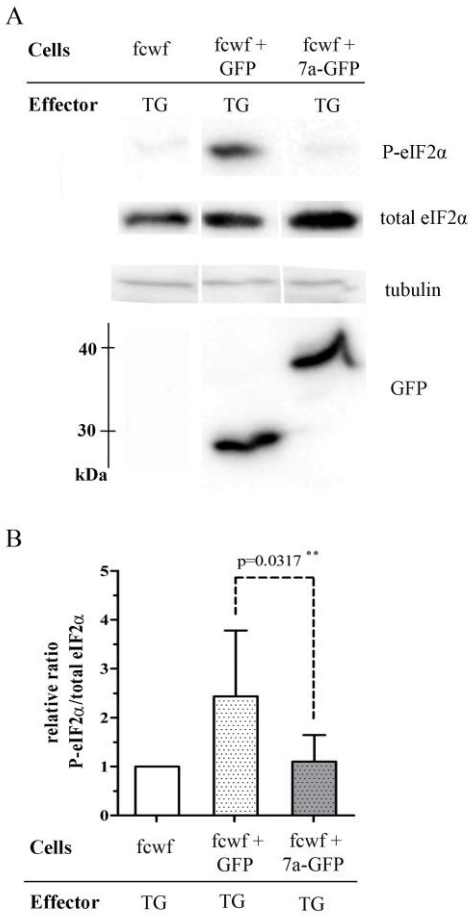

Figure 5.2: Expression of protein 7a causes suppression of TG induced eIF2α phosphorylation. (A) Western blot detection for total and phosphorylated eIF2α in fcwf, fcwf-GFP and fcwf-7a cells. Tubulin detection was included as a loading control and GFP detection was included as control for recombinant protein expression. (B) Graphical representations of P-eIF2α/total eIF2α ratios of transduced cells normalized against untransduced cells ratios (five independent replicates). Asterisks indicate statistically significant differences ($p \leq 0.05$).

5.3.2 Deletion of both ORF3 and ORF7 from FIPV induces increased levels of phosphorylation of eIF2α

Since protein 7a of FIPV 79-1146 reduces P-eIF2α levels induced by the external stress inducer thapsigargin, we investigated if FIPV-wt (expressing 7a) prevented infection-induced phosphorylation of eIF2α and if deletion of ORF7 would affect this. Therefore, fcwf cells were infected with either FIPV-wt or FIPV-Δ7 and lysates were collected 18hpi. Western blotting was performed for total and phosphorylated eIF2α. Infection of fcwf cells with FIPV-wt resulted in an only minor, non-significant increase in eIF2α phosphorylation levels compared to mock infected cells (Figure 5.3A, lanes 1-2; Figure 5.3B, columns 1-2). Somewhat surprisingly, in comparison with FIPV-wt, eIF2α phosphorylation was not significantly increased upon infection of fcwf cells with FIPV-Δ7 (p=0.3048) (Figure 5.3A, lanes 2-3; Figure 5.3B, columns 2-3).

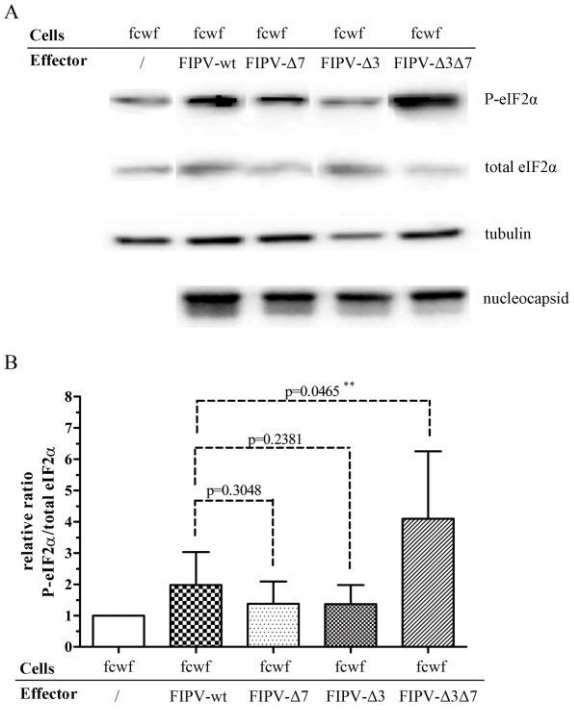

Figure 5.3: Induction of eIF2α phosphorylation by infection of fcwf cells. (A) Western blot detection for total and phosphorylated eIF2α. Tubulin detection was included as a loading control and nucleocapsid detection was included as control for infection. (B) Graphical representations of P-eIF2α/total eIF2α ratios of infected samples normalized against ratios in mock cells of four independent replicates. Asterisks indicate statistically significant differences ($p \leq 0.05$).

This led to the following hypotheses: (i) proteins expressed from ORF7 are not important to impede eIF2α phosphorylation during FIPV-wt infection, or (ii) there are additional viral proteins of FIPV-wt, next to protein 7a, that antagonize the phosphorylation of eIF2α in a

redundant way. To examine if viral proteins encoded by the other accessory ORF3 could have this latter potential, eIF2α phosphorylation levels were investigated upon infection with FIPV-Δ3 or FIPV-Δ3Δ7 (Figure 5.3). P-eIF2α levels in FIPV-Δ3-infected cells were comparable with FIPV-wt induced levels (p=0.2381). In contrast, FIPV-Δ3Δ7 infection resulted in a significantly increased phosphorylation of eIF2α (p=0.0465). This result implicated that both ORF3 and ORF7 encoded protein(s) have redundant antagonizing functions in the eIF2α phosphorylation antiviral pathway.

5.3.3 Protein 7a reverses the FIPV-Δ3Δ7 induced phosphorylation of eIF2α

The results described above lead to the conclusion that protein 7a could represents one of the eIF2α phosphorylation antagonists during FIPV-wt infection. To further confirm this, eIF2α phosphorylation levels were evaluated after protein 7a was administrated in trans to FIPV-Δ3Δ7. Thus, fcwf-7a cells were infected with either FIPV-wt or FIPV-Δ3Δ7 and lysates were collected at 18hpi. Immunoblotting revealed that, in cells expressing protein 7a, activation of eIF2α phosphorylation by FIPV-Δ3Δ7 was comparable to FIPV-wt (p=0.2) (Figure 5.4). Hence, expression of protein 7a was sufficient to reverse the phosphorylation induction of eIF2α by FIPV-Δ3Δ7.

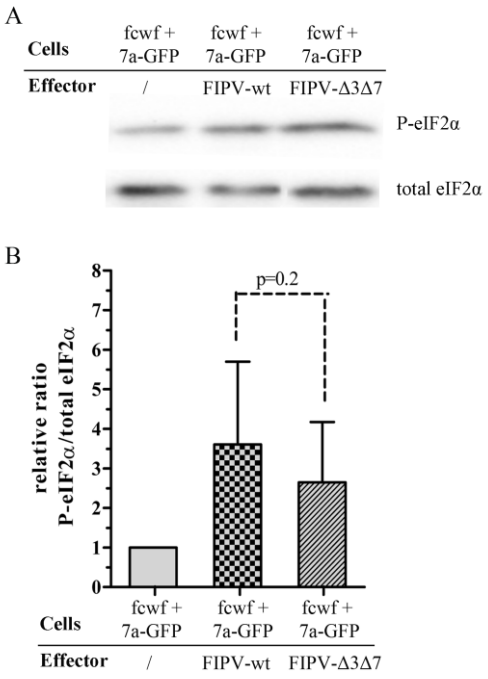

Figure 5.4: Protein 7a suppressed the eIF2α phosphorylation induced by FIPV-Δ3Δ7. (A) Western blot detection for total and phosphorylated eIF2α of fcwf-7a cells infected with FIPV-wt and FIPV-Δ3Δ7. (B) Graphical representations of P-eIF2α/total eIF2α ratios of infected samples normalized against mock ratios of three independent replicates.

5.3.4 Deletion of the predicted PP1c binding motif reduces the eIF2α dephosphorylation capacity of protein 7a

Decreased phosphorylation levels of eIF2α during FIPV infection could be the consequence of either (i) a direct inhibition of kinase (PKR) activation, through for instance, sequestering dsRNA, inhibiting PKR dimerization or PKR degradation, or (ii) an indirect counteraction of the kinase function through activation/recruitment of antagonist phosphatases (e.g. PP1) (Garcia et al., 2007; Langland et al., 2006). The sequence homology of FIPV protein 7a with TGEV protein 7, together with the presence of a conserved PP1c binding motif, are strong indicators that FIPV protein 7a indirectly leads to dephosphorylation of eIF2α with the help of PP1 (Cruz et al., 2011). This mode of action was further explored by creating a recombinant protein 7a in which the consensus PP1c binding motif (RVIF) was deleted (7a-PP1cDEL-GFP). The effect of this deletion on eIF2α phosphorylation was evaluated both in the context of stress induction by thapsigargin and FIPV infection.

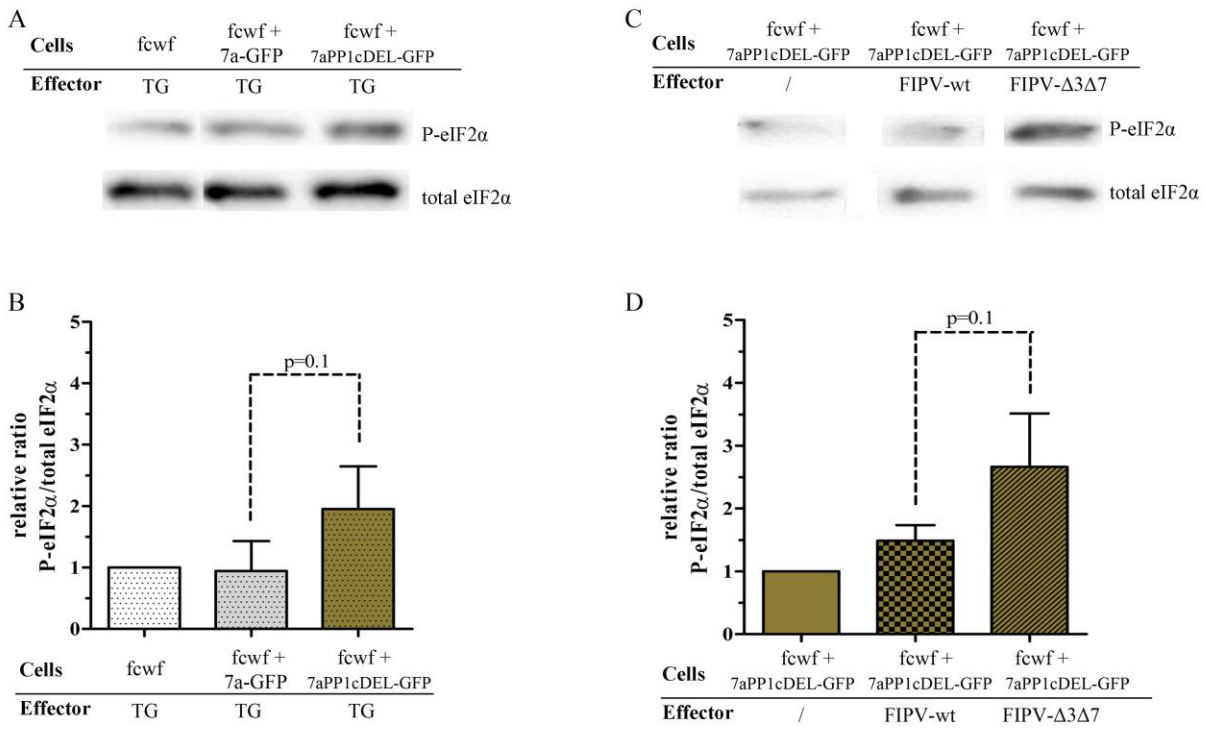

Figure 5.5: Effect of PP1-mutated protein 7a on eIF2α phosphorylation. (A,B) Treatment of fcwf-7a cells and fcwf-7aPP1cDEL cells with thapsigargin. (C,D) Infection of fcwf-7aPP1cDEL cells with FIPV-wt and FIPV-Δ3Δ7**.** (A, C) Western blot detection for total and phosphorylated eIF2α. (B, D) Graphical representations of P-eIF2α/total eIF2α ratios of transduced or infected cells normalized against untransduced or mock ratios, respectively, of three independent replicates.

The phosphorylation levels in fcwf-7aPP1cDEL cells after thapsigargin treatment were higher than the levels in fcwf-7a cells, however significance level of 0.05 was just not reached (p=0.1). This result indicates that the mutated protein 7a could no longer reduce the thapsigargin-induced phosphorylation levels of eIF2α, which is in contrast to the wt protein 7a. (Figure 5.5A and B). Furthermore, providing protein 7a-PP1cDEL-GFT in trans to FIPV-Δ3Δ7 may no longer decrease P-eIF2α/eIF2α ratios in contrast to the parental protein 7a, as a tendency toward elevated phosphorylation levels of fcwf-7aPP1cDEL infected with FIPV-Δ3Δ7 was seen compared to the levels in those cells infected with FIPV-wt (p=0.1) (Figure 5.5C and D). These data suggest that protein 7a interferes with eIF2α phosphorylation via PP1.

5.4 Discussion

Suppression of translation is one of the key elements of innate responses of eukaryotic cells to viral infection. In many cases, the global protein synthesis is shut down in infected cells via phosphorylation of eIF2α, which represents one of the best studied IFN-induced antiviral pathways (Clemens, 1997; Garcia et al., 2007; Kaufman, 1999). Feline coronaviruses, like all other viruses, entirely rely on the host cell translation machinery for synthesis of their structural and functional proteins. Therefore, it is likely that these viruses have evolved mechanisms that counteract suppression of translation, including phosphorylation of eIF2α, as has been described for numerous animal viruses (Gale and Katze, 1998; Hengel et al., 2005; Katze et al., 2002).

Results presented in this study demonstrate that both FIPV accessory ORFs, ORF3 and ORF7, appear to be involved in the FIPV-induced suppression of eIF2α phosphorylation. The present study focused on one protein expressed from ORF7, namely protein 7a, because we previously linked this protein to IFN-α antagonism (Dedeurwaerder et al., 2014). Furthermore, protein 7a of FIPV is an orthologue of protein 7 of TGEV, which has already been demonstrated to be involved in eIF2α dephosphorylation and overall interference with the innate immune response both *in vitro* and *in vivo* (Cruz et al., 2013; Cruz et al., 2011).

We found that protein 7a, expressed in the absence or presence of other FIPV encoded proteins, inhibits phosphorylation of eIF2α. Moreover, it appears that a deletion of the conserved amino acids representing the predicted PP1c binding motif in protein 7a, presumably abolished its eIF2α dephosphorylation capacity. However, this latter mechanism could just not been proven significantly (p=0.1). This could be attributed to the small sample

size (three replicates) and the semi-quantitative analysis method, which left us with high variation rates, making it difficult to reach a significant level of 0.05 (Noymer, 2008)

It is generally accepted that the accessory proteins of coronaviruses play an important role in virulence, as deletion of the ORFs encoding for them lead to attenuated phenotypes (de Haan et al., 2002; Haijema et al., 2004; Ortego et al., 2003). Nevertheless, only recently, researchers have shed some light on their functions. Especially for feline coronaviruses, data on the functional analysis of the accessory proteins is very scarce. To our knowledge, the findings that (i) mutations in the 3c gene are linked to FIPV pathotype, (ii) the presence of ORF7 is crucial for efficient replication in monocytes/macrophages, (iii) the secretion of the small glycoprotein 7b might suggest a signaling role, and (iv) protein 7a of FIPV 79-1146 is able to interfere with IFN-induced replication restriction, are the main findings regarding the role and importance of feline coronavirus accessory proteins (Chang et al., 2010; Dedeurwaerder et al., 2013; Dedeurwaerder et al., 2014; Pedersen et al., 2012; Rottier, 1999).

Zooming in on one of the IFN-induced antiviral pathways, the eIF2α phosphorylation, we were able to unravel an important role for ORF3 and ORF7 in impeding this pathway, as a mutant FIPV deleted in both ORFs (FIPV-Δ3Δ7) induced significant higher eIF2α phosphorylation levels compared to FIPV-wt. Protein(s) expressed from ORF3 and ORF7 seem to execute a redundant role, as both ORFs could compensate for each other, illustrated by the fact that FIPV-Δ3 and FIPV-Δ7 did not induce higher eIF2α phosphorylation levels.

In general, both DNA and RNA viruses appear to have evolved many mechanisms designed to defeat the shutoff of protein synthesis resulting from the phosphorylation of eIF2α (Cassady et al., 1998; Cruz et al., 2011; Garcia et al., 2007; Langland et al., 2006; Wang et al., 2009; Ye et al., 2007). Moreover, the expression of a viral protein that substitutes another viral protein, which eventually results in the same antagonizing effect, has already been demonstrated for several viruses. For instance, in the case of vaccinia virus (VV), two viral proteins (K3L and E3L) are employing different pathways to antagonize PKR activation, acting as a PKR pseudosubstrate or a high affinity dsRNA-binding protein, respectively (Carroll et al., 1993; Davies et al., 1993). HSV-1 encodes at least 4 proteins that all result in the inhibition of IRF-3 activation, a transcription factor involved in type I IFN production (Melchjorsen et al., 2006; Melroe et al., 2007; Peri et al., 2008; Verpooten et al., 2009). Also the IFN downstream signalling is targeted by at least two HSV-1 encoded proteins, either by binding to PKR (US11) or by bridging eIF2α and PP1 (γ34.5), thereby facilitating the specific dephosphorylation of eIF2α (He et al., 1997; Poppers et al., 2000). Moreover, this property is not limited to DNA viruses, as for instance coronaviruses, like IBV (infectious

bronchitis virus) have also been reported to target different levels of the eIF2α phosphorylation pathway, including induction of GADD34 expression and inhibition of PKR autophosphorylation by the dsRNA binding protein nsp2 (Wang et al., 2009). The ability to modulate a single IFN-induced antiviral pathway by different viral proteins, renders the virus more resistant to the high selection pressure in a host environment. Indeed, even when the expression of one viral gene or function of one viral protein is negatively affected by mutations or host antagonizing activities, the virus can still utilize the other protein(s) to result into the same effect.

This also is the first report that links ORF3 with suppressed eIF2α phosphorylation. The exact mode of action of ORF3 encoded proteins in this respect has not been explored in this study, but is certainly of interest for further research. Possible mechanisms are (i) the direct interaction with PKR, resulting in the irreversible blocking of PKR or interference with the PKR activation site (cf. HSV-1 US11 protein (Poppers et al., 2000)), (ii) a dsRNA binding capacity, capturing in that way the important PKR activators (cf. VV E3L (Davies et al., 1993)) or (iii) direct or indirect dephosphorylation of eIF2a (cf. HSV-1 γ34.5 (Li et al., 2011b)). Limited, non-relevant similarities were found between the sequences of protein 3a and 3b and the non-redundant protein sequence and UniprotKB/Swissprot database maintained by NCBI using the Basic Local Alignment Search Tool (Blastp). However, more detailed sequence/conserved domain alignments and topology predictions using more sophisticated programs, may indicate if the mechanism employed by FIPV ORF3 encoded proteins are similar as for other IFN-antagonistic proteins or are totally new. It would also be interesting to check if the proteins encoded by type I FCoV ORF3 have the same properties, as regions within the ORF3 genes are less conserved between the two FCoV pathotypes due to exchange of this ORF (together with S gene) between type I FCoV and canine coronavirus (CCoV) in type II FCoVs.

We reported earlier that protein 7a, one of the proteins expressed from ORF7 of FIPV 79-1146 is an IFN-α antagonist, as it can nullify the negative effects of IFN-α pretreatment on FIPV-Δ7 replication (Dedeurwaerder et al., 2014). Here, we find that protein 7a is involved in suppressing phosphorylation of eIF2α. Examining its mode of action in more detail, revealed that protein 7a probably counteracts the eIF2α phosphorylation via the PP1c complex. A similar mechanism has recently been described for another alphacoronavirus 1 accessory protein, namely protein 7 of TGEV, as well as for several DNA virus proteins; including HSV-1 γ34.5, human papillomavirus (HPV) E6 protein and African swine fever virus (ASFV) DP71L; and cell proteins like mammalian GADD34 proteins (Brush et al.,

2003; Cruz et al., 2011; He et al., 1997; Kazemi et al., 2004; Li et al., 2011b; Zhang et al., 2010). All these proteins, including protein 7a of FIPV, have a highly conserved C-terminal sequence in common, which is identified as the PP1c binding motif (R/K)VxF. It has been demonstrated that this motif is sufficient for protein phosphatase 1c (PP1c) binding (Ajuh et al., 2000; Hsieh-Wilson et al., 1999; Schillace et al., 2001). PP1c dephosphorylates a wide range of substrates *in vitro*, but interaction with targeting proteins enhances specificity, permitting PP1c to dephosphorylate only those substrates in the neighbourhood of the targeted complex, being in this case eIF2α (Cohen, 2002). The interplay of protein 7a and ORF3 encoded proteins with the eIF2α phosphorylation that was suggested by our results is summarized in a hypothetical model represented in Figure 5.6.

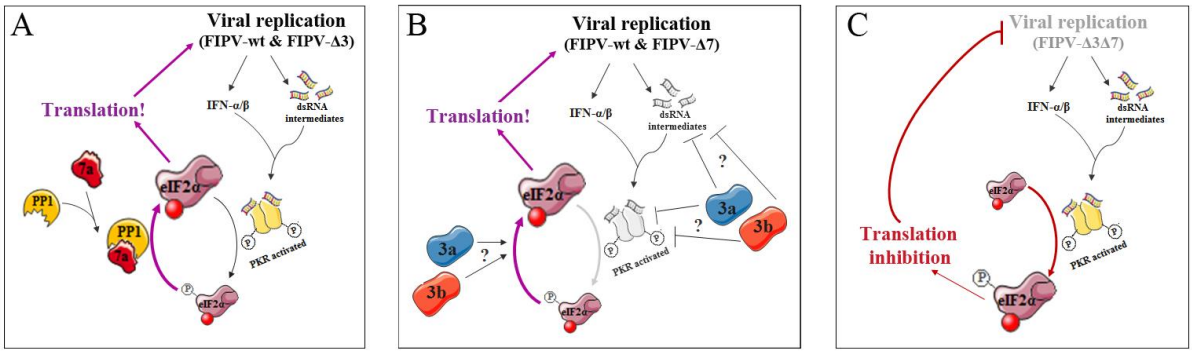

Figure 5.6: Hypothetical model for the eIF2α phosphorylation counteraction by FIPV encoded accessory proteins 7a, 3a and 3b. (A) Viral infection and antiviral response in the presence of a functional protein 7a (cf. FIPV-wt and FIPV-Δ3). (B) Viral infection and antiviral response in the presence of functional ORF3 encoded proteins 3a and/or 3b (cf. FIPV-wt and FIPV-Δ7). (C) Viral infection and antiviral response in the absence of functional protein 7a or ORF3 encoded proteins 3a and/or 3b (cf. FIPV-Δ3Δ7).

In conclusion, in FIPV-infected cells, protein 7a counteracts PKR by modulating eIF2a activity. Probably, protein 7a of FIPV is counteracting the PKR activation in the same way as described for its orthologue protein 7 of TGEV, by the recruitment of PP1, which is of great importance to the dephosphorylation reaction. In addition, we showed that ORF3 of FIPV encodes for a protein(s) with a redundant function in suppressing phosphorylation of eIF2α.

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Chapter 6

General discussion

Feline coronaviruses occur as two distinct pathotypes. Feline enteric coronavirus (FECV) seems to be confined mainly to the intestinal tract and causes mild, often unapparent, enteritis. By efficiently infecting macrophages and monocytes, feline infectious peritonitis viruses (FIPVs) can escape from the gut and cause a lethal systemic disease with multi-organ involvement (Chang et al., 2010; Pedersen, 2009). Thus, the monocyte/macrophage lineage represents a major cellular reservoir for FIPV. Paradoxically, the prime functions of these immune cells is to organize host defences against invading pathogens in order to destroy them. They support inflammation through the secretion of autocrine and paracrine cytokines. One such cytokine, IFN-α, induces significant antiviral activities that affect the ability of many viruses to infect and replicate in the target cell. The high pathogenicity of FIPV suggests that this virus has evolved mechanisms to overcome the IFN-induced antiviral immune response.

Viral genomes are constantly subjected to a high selective pressure. As a consequence, only those genes that confer a biological advantage to the virus in the natural host environment are maintained. Hence, it is generally accepted that the group-specific (i.e. accessory) genes of coronaviruses (CoVs) contribute to the viral fitness during virus-host interactions. This notion is supported by the fact that viruses with deletions in the accessory ORFs are attenuated (de Haan et al., 2002; Haijema et al., 2004; Narayanan et al., 2008; Ortego et al., 2003). Notwithstanding their importance, the exact functions of these genes are still poorly understood. Currently, there are a range of proposed functions for accessory proteins of other CoVs, including modulation of viral replication, as well as acting as apoptosis inducers and IFN antagonists, to name a few (Cruz et al., 2011; Koetzner et al., 2010; Kopecky-Bromberg et al., 2007; McBride and Fielding, 2012; Minakshi et al., 2009; Narayanan et al., 2008; Niemeyer et al., 2013).

Inspired by this knowledge, it was explored in this PhD thesis if the FIPV encoded accessory proteins contribute to virus replication in monocytes (Chapter 3) and if their presence antagonizes the antiviral responses induced by IFN-α (Chapter 4 and 5). Such knowledge may form a significant basis towards vaccine development and clinical treatment of FCoV infections.

Monocyte/macrophage tropism of FIPV

The ability to replicate and sustain its infection in monocyte/macrophages is the dominant factor that enables FIPV to result in the typical immunopathological damage (Dewerchin et al., 2005; Kipar et al., 2005; Rottier et al., 2005; Stoddart and Scott, 1989). In the last decade,

research groups have extensively studied the macrophage tropism of FIPV. In 2005, it was proven by Rottier *et al.* that type II FIPV infection of macrophages was governed by the C-terminal domain of the spike protein. As this domain is responsible for membrane fusion, they correlated this fusion property with the cell tropism. Thus, the spike protein is controlling macrophage infection at least at the level of cell entry (Rottier et al., 2005). Recently, sequence comparison between field type I FIPV and FECV confirmed the involvement of the fusion peptide in the macrophage tropism. This sequencing analysis identified two alternative amino acid differences in this fusion domain, which together distinguish FIPV from FECV in >95% of the cases (Chang et al., 2012). A similar approach was used to compare the furin cleavage sequence site in the S1/S2 region of the spike protein between type I FIPV and FECV and revealed an additional biotype relevant mutation (Licitra et al., 2013). Nevertheless, if these mutations in the spike protein would be the sole determinant for efficient growth in macrophages, one would expect FIPV to occur far more frequently. Research done at our laboratory showed also that type II FECV was still able to enter and replicate in monocytes, but that, contrary to FIPV, this replication decreased 12hpi, suggesting also discriminating factors post cell entry (Dewerchin et al., 2005). So it is generally believed that additional mutations in other viral proteins are required for the tropism switch. Indeed, alterations in the accessory genes might also favour systemic dissemination (Balint et al., 2012; Chang et al., 2010; Herrewegh et al., 1995; Pedersen et al., 2012; Vennema et al., 1998). The 3c protein may play a particular role in this respect, since it is essential for replication of FCoV in the gut, but becomes nonessential when infecting macrophages. Inactivation of the 3c protein may even enhance virus fitness for and subsequent replication in monocytes/macrophages and prevent FIPV from returning to the intestine, explaining the rare outbreaks of FIP (Balint et al., 2012; Chang et al., 2010; Chang et al., 2012; Pedersen et al., 2012).

To evaluate the importance of the accessory genes during replication in monocytes/macrophages, we compared the infection kinetics of FIPV-wt with three accessory ORF deletion mutants of this virus, designed FIPV-Δ3 (deleted in ORF3), FIPV-Δ7 (deleted in ORF7) and FIPV-Δ3Δ7 (deleted in both ORF3 and 7). The results demonstrated that ORF7 is indispensable for sustained virus replication and production in peripheral blood monocytes. The deletion of ORF3 had only a minor negative impact on the efficiency of replication, which could be mapped to the absence of protein 3a and/or 3b, as protein 3c was both mutated in the wild type FIPV and FIPV-Δ3, something that is in agreement with the earlier hypothesis that protein 3c is not important for macrophage

infection, as described above. The overall impact of deleting these accessory ORFs was seen from 12hpi. This, together with the fact that there is no evidence for a structural character of the FCoV accessory proteins, indicates that FIPV infection of monocytes is governed by the accessory proteins acting most likely at replication steps subsequent to cell entry. As discussed above, the latter property is more likely controlled by the spike protein. An infection block after cell entry (e.g. before or at translation level) could also explain the resistance to FIPV infection of some cats, as suggested by Dewerchin et al. (2005). The ability of inducing a strong antiviral immune response, which could block virus infection at different steps of the replication cycle (pre, during and post cell entry), could be one of the host factors determining the intrinsic variability in susceptibility to FIPV. Seen the central importance of type I interferons, and specifically IFN-α in antiviral defence, in this thesis, further focus was laid on IFN-α induced immune responses (Garcia-Sastre and Biron, 2006; Goodbourn et al., 2000; Grandvaux et al., 2002; Muller et al., 1994).

IFN-α dependent control of FIPV replication in monocytes/macrophages

ORF7-dependent replication of FIPV is seen both in monocytes/macrophages and fcwf cells (macrophage-like cells) but not in CrFK cells (epithelial cells) (data not shown), suggesting that ORF7 proteins are functioning on typical macrophage pathways, or at least a pathway that is not active/activated in CrFK cells. Testing both cell lines during the optimisation of the IFN-α detection bioassay, revealed that CrFK cells were far less sensitive to recombinant feline IFN-α than fcwf cells, suggesting that the IFN-α induced antiviral response may indeed be one of the pathways that are differentially activated in these two cell lines. In addition, this IFN-α bioassay also demonstrated that FIPV infected monocytes already produced IFN in the supernatant at 4hpi, while IFN was only detected at 18hpi in supernatant of infected fcwf cells. Therefore, it appears that purified peripheral blood monocytes more rapidly and strongly induce IFN, which perhaps may contribute to the lower replication and slower growth kinetics of FIPV in these cells compared to fcwf cells. This observation strengthens the hypothesis launched by Van Hamme *et al*., namely that the low percentage of monocyte infection may not be due to a lack of FIPV internalisation, as a majority of the cells takes up virus particles, but may be due to a cellular mechanism that restricts infection at a level post virus uptake. They suggested that at least the capacity of a cell to mediate FIPV uncoating may characterize in part the susceptible subpopulation of monocytes. Nevertheless, other restrictions could not be excluded (Van Hamme, 2009). Our results may propose that also the IFN-induced antiviral response could be another determining factor for FIPV susceptibility of

monocytes. Although speculative, it is possible that FIPV is only able to establish a productive infection in a subtype of monocytes/macrophages that do not rapidly produce high levels of IFN and/or inefficiently induce an antiviral state. This hypothesis is supported by a phenomenon observed for SARS-CoV infection, as SARS-CoV proteins were also only detected in monocytes/macrophages that did not produce significant amount of IFN-α (Yilla et al., 2005). Identifying putative FIPV sensitive monocyte subtype(s) or identifying the factors that contribute to replication of FIPV in only a minor fraction of monocytes would represent a big step forward in our understanding of FIP pathogenesis.

Antagonistic properties of FIPV against the innate immune response

Like all viruses, the moment FCoVs enter the host, they have to cope with a powerful immune defence, both the innate immune response and later the adaptive immune response. Research on the adaptive immunity has revealed that cats are protected against FIP development, when they are able to establish a strong cellular immune response (de Groot-Mijnes et al., 2005; Pedersen and Black, 1983). In contrast, the humoral immune response is not protective for the host and even may contribute to disease, as FCoVs has been reported to efficiently evade and even abuse this response (Cornelissen et al., 2007, 2009; Dewerchin et al., 2006; Hohdatsu et al., 1991; Olsen et al., 1992; Paltrinieri et al., 1998; Takano et al., 2008). Despite intensive research on adaptive immune responses, limited information is available on the early innate immune activity against FCoV infections. Type I interferon (IFN-α/β), a key modulator of innate antiviral defence, elicits multifaceted effects that are characterized by the expression and antiviral activity of interferon-stimulated-genes (ISGs). The purpose of our research was to investigate whether IFN-α may be an important determinant during FIPV infections and if FIPV encodes mechanisms to counteract the antiviral power of IFNs.

We demonstrated that **FCoV infection induced IFN-α**. Both in the sera of FIP cats and in the supernatants of FIPV infected fcwf cells and peripheral blood monocytes, significant quantities of IFN were detected. IFN was also found in the sera of FECV infected cats, especially early after infection. This IFN was not directly produced by infected enterocytes themselves, as no IFN could be detected in the supernatants of ileocyte and colonocyte cell cultures (established by Desmarets *et al.*, 2013) infected with two different strains of type I FECV (data not shown). In line with a TGEV infection, we can speculate that intra-epithelial IFN-α producing leukocytes may be responsible for the secretion of these high amounts of IFN-α, following a short contact with viral structures (Fitzgerald-Bocarsly, 1993; Riffault et

al., 2000). Whether these cells or others account for the IFN production during an FECV infection would be interesting to investigate further.

The fact that IFN is produced during active FCoV infection, suggests that FCoVs are able to replicate to some extent in cells that are primed to induce an antiviral state via IFN-α, indicating that FCoVs may have established mechanisms to evade the IFN-α induced antiviral signalling cascade. In other coronaviruses, these mechanisms are often regulated by non-structural and group-specific proteins that circumvent the IFN signalling pathway or interferon stimulated gene (ISG) activities (Cruz et al., 2011; Koetzner et al., 2010; Kopecky-Bromberg et al., 2006; Ye et al., 2007). Until now, no IFN-interfering proteins have been identified for FCoVs. As all coronaviruses have the same structural appearance, genome constellation and replication strategy, it may be assumed that they all target the IFN system in the similar ways. However, the group-specific proteins, also designed as accessory proteins, of feline coronaviruses share no sequence homology with proteins of other coronavirus genera (β, γ and δ), indicating that the specific (sub)groups within this family have evolved their own characteristic functions. Except for the fact that the accessory proteins within the alphacoronavirus group are important for virus virulence *in vivo*, limited information is available on the function of these proteins from this virus genus (Haijema et al., 2004; Ortego et al., 2003). One research team described that accessory protein 7 of TGEV counteracts the function of two major interferon-stimulated-genes (Cruz et al., 2013; Cruz et al., 2011). This protein shows 70% amino acid homology with protein 7a of FIPV, strongly suggesting a similar role for the latter accessory protein of FIPV. In addition, it appears that it is very beneficial for FCoV fitness and survival to conserve gene 7a, as it is over 90% identical among FCoV patho- and serotypes. As we observed that ORF7, which encodes for this protein, is of main importance for efficient replication in cells, we further examined the potential role of **protein 7a as an interferon antagonist**.

Our results showed that FIPVs deleted in ORF7 were significantly more sensitive to feline IFN-α treatment in comparison with FIPV-wt. We further demonstrated that protein 7a was the main determinant for IFN-α interference. However, we found that this protein requires the ORF3 encoded accessory proteins 3a and/or 3b to fully reverse the IFN antiviral response on replication. The presence or absence of all of these accessory proteins has no influence on IFN-α production, which suggests a counteraction with the IFN- mediated antiviral effects instead. Our results strongly suggest that both proteins are not functioning in a synergetic manner as deleting both ORFs did not result in a stronger replication inhibition post-IFN treatment than deleting either ORF3 or ORF7. This indicates that protein 7a and ORF3

proteins either regulate each other, affecting one or more IFN checkpoint(s) or that they both act on similar IFN antiviral pathways, such as protein translation inhibition. Whether the cooperation between protein 7a and protein 3a and/or 3b is characterized by a direct interaction between both proteins is speculative at this point, and has to be further examined. A direct interaction between non-structural coronaviral proteins would not be the first that is described. For instance, studies have shown that SARS-CoV accessory ORF6 protein is interacting with nsp8, a second and newly identified RNA-dependent RNA polymerase for SARS-CoV, supporting a regulating role for ORF6 in virus replication (Kumar et al., 2007). Furthermore, accessory protein 7a of SARS-CoV is interacting with several viral proteins, including accessory proteins 3a and 8b, although the biological relevance of these interactions is still not well understood (Keng et al., 2006; Tan et al., 2004).

It is not clear yet which of the multiple ISG products act against FIPV infection, either *in vivo* or in cell cultures. One of the most important IFN-induced antiviral pathways is the **phosphorylation of eukaryotic translation initiation factor eIF2α**. EIF2α is one of the key players driving protein synthesis in eukaryotic cells by mediating the binding of $tRNA^{met}$ to the ribosome in a GTP-dependent manner. It can be phosphorylated by a number of protein kinases which are activated upon sensing cell stress, such as amino acid starvation (GCN2), ER stress (PERK), the presence of dsRNA (PKR) or heme deprivation (HRI). This phosphorylation inhibits the recycling of an active GTP-bound eIF2α and subsequently inhibits translation (Kaufman, 1999; Proud, 2005). Viral replication mainly activates PKR through the generation of dsRNA and induction of IFN. Because viruses rely on the host cell translation machinery for their viral protein synthesis, many viruses have adopted a massive array of different strategies to inhibit PKR or the subsequent phosphorylation of eIF2α (Gale and Katze, 1998; Hengel et al., 2005; Katze et al., 2002). In line with this, we observed that FIPV infections do not induce high phosphorylation levels of eIF2α in fcwf cells, supporting the hypothesis that FIPV evolved immune evasive strategies against this antiviral pathway. Moreover, we demonstrated that the presence of either ORF3 or ORF7 is essential to sustain this specific antagonizing property. This strongly suggests that these ORFs use redundant forces resulting in the same effect, namely dephosphorylation of eIF2α and thus, allowing translation initiation. Several viruses, including VV, HSV-1 and IBV, are using this strategy to lower the high antiviral pressure induced by the host (Carroll et al., 1993; Davies et al., 1993; He et al., 1997; Poppers et al., 2000; Wang et al., 2009).

During this thesis, we further focussed on protein 7a because unravelling its mode of action as an IFN-antagonist was our initial goal. Furthermore, our interest in revealing the specific

function of protein 7a was also stirred up because of its high sequence homology with the TGEV IFN-antagonistic protein 7. We found that FIPV **protein 7a was able to modulate eIF2α phosphorylation** induced either by a viral-independent stressor (thapsigargin) or by viral infection, indicating that the PKR/eIF2α system is one of the targets of protein 7a. We also found that the presence of an intact predicted phosphatase protein 1c (PP1c) binding motif was essential to execute this function. We hypothesize that protein 7a uses this motif to escort PP1 to the eIF2α pathway. PP1 belongs to the class of serine/threonine phosphatases and can regulate different processes by complexation of the PP1 catalytic subunit to various regulatory subunits, which determine the substrate specificity and compartmentalisation. Co-localisation staining and co-immunoprecipitations should be performed to confirm if protein 7a is directly interacting with PP1, and in that way recruits PP1 to dephosphorylate eIF2α.

The exact mode of action of ORF3 encoded proteins on eIF2α dephosphorylation has not been explored in depth in this thesis, but is certainly of interest for further research. Possible mechanisms are (i) a direct interaction with PKR, resulting in the irreversible blocking of PKR or interference with the PKR activation site [cf. HSV-1 US11 protein (Poppers et al., 2000)], (ii) direct or indirect dephosphorylation of eIF2α [cf. HSV-1 γ34.5 (Li et al., 2011)] or (iii) a dsRNA binding capacity, in this way capturing this critical viral PKR activator [cf. VV E3L (Davies et al., 1993)]. Sequence alignment using Blastp 2.2.28 did not reveal relevant similarities with earlier described IFN-antagonistic proteins, indicating that FIPV ORF3 encoded proteins may either use another, yet unidentified, mechanism or possess other, yet unrevealed, structural and/or functional motifs.

Although we demonstrated that protein 7a plays a role during the dephosphorylation of eIF2α, this IFN-induced antiviral signalling pathway cannot explain the higher susceptibility of FIPV-Δ7 to IFN-α treatment (Dedeurwaerder et al., 2014), as it appears that ORF3 encoded protein(s) are also PKR antagonists that can compensate for protein 7a. Our results suggest that protein 7a, in cooperation with ORF3 proteins, may also target other IFN-induced pathways. This hypothesis is not unimaginable, as also TGEV protein 7 appears to have multiple IFN-antagonizing properties, counteracting with both PKR and RNaseL activation (Cruz et al 2011). Moreover, many viral proteins have been described to have multiple IFN-antagonizing functions. For instance, ORF6 of SARS-CoV suppresses the production of IFN as well as the induction of IFN signalling pathways (McBride and Fielding, 2012) and also the E6 protein of human papillomavirus inhibits both IFN JAK/STAT-signalling as eIF2α phosphorylation (Kazemi et al., 2004; Li et al., 1999). This is in line with the fact that the IFN-induced antiviral response is regulated by more than PKR

activation alone, thus, viruses should antagonize different IFN-activated pathways in order to sustain their infection (Abraham et al., 1999; Goodbourn et al., 2000; Yang et al., 1995). Getting insights into possible other functions of protein 7a would help to reveal the full picture.

Summarizing, expression of FIPV proteins 7a and ORF3 hampers the development of an efficient IFN-dependent antiviral state. Notwithstanding that both antagonize eFI2α phosphorylation, this property cannot solely explain the higher IFN resistance of FIPV-wt compared to FIPV-Δacc. Our data suggest that the accessory proteins of FIPV use complex and multifaceted mechanisms to counteract the IFN-induced antiviral response from which different parts still need to be investigated. These findings have led to a hypothetical model for the type I IFN-antagonistic properties of these FIPV accessory proteins, depicted in Figure 6.1.

Link between FCoV-IFN interplay and *in vivo* pathogenesis

By showing that FIPV-wt is able to overcome the antiviral effects of IFN at least to some extent, our results could partly explain the overall marginal efficacy of IFN treatment of FIP cats (Hartmann and Ritz, 2008; Weiss et al., 1990). Moreover, a significant concentration of IFN-α was detected in the serum of FCoV infected cats, which was unable to prevent the virus from inducing fatal disease. However, it cannot be excluded that FIPV infected cells may escape this *in vivo* IFN pressure by extravasating into the organ tissues, where local IFN concentrations may be low. Staining of pyogranulomas, collected from FIP cats, for IFN-α and its receptor could give us a decisive answer on this, but until now no feline specific antibodies are available.

It is generally known that IFN-α is a potent modulator of overall cellular immune responses by upregulating MHC-I molecules, promoting IFN-γ expression and improving NK activity (Goodbourn et al., 2000; Reiter, 1993; Samuel, 2001). FIPV infections are characterized by a suppressed lymphocyte proliferation, lack of IFN-γ production and reduced NK cytotoxicity, confirmed by our research group and others (Gunn-Moore et al., 1998; Haagmans et al., 1996; Takano et al., 2007; Vermeulen, 2013; Vermeulen et al., 2013). Next to the ISG evasive potential of FIPV shown in this thesis, it would be interesting to investigate if FIPV is also able to evade immune-modulating effects of IFN-α, which could help to explain the crippled cellular immune response observed in FIP cats.

FCoV infection is characterized by a chronic nature, restricting its replication to a low percentage of cells and persisting for a couple of months. Our results show that FIPV

infection is inducing IFN and the virus is not able to fully impede the IFN antiviral activity, as FIPV-wt encountered also some replication restriction due to IFN pre-treatment. These facts could contribute to the typical character of FIPV infection.
Last but not least, the fact that a combination of ORF3 and 7a proteins is needed to protect feline coronaviruses from IFN-induced antiviral activity can also provide a likely explanation for the high attenuation of the FIPV-Δacc mutants with respect to FIPV-wt when inoculated into cats (Haijema et al., 2004).

Main conclusions that can be drawn from this thesis

- Deletion of the accessory ORFs (ORF3 and ORF7) from type II FIPV has a negative influence on the virus replication efficiency in peripheral blood monocytes and macrophage-like fcwf cells
- In contrast to FIPV-wt, FIPVs deleted in ORF7 could no longer sustain its replication in monocytes after 12hpi
- The CrFK cell line has substantial drawbacks when addressing FIPV infection and FIPV protein functionality
- FIPV infection induces IFN-α production
- FIPV is still able to replicate in the face of IFN-α and at least the presence of accessory protein 7a and ORF3 is needed
- Accessory protein 7a of FIPV can impede both ER-stress and infection-induced eIF2α phosphorylation, most probably by the recruitment of protein phosphatase 1 (PP1)
- ORF3 encoded proteins of FIPV can antagonize the eIF2α phosphorylation, but their exact mode of action needs to be elucidated
- ORF3 encoded proteins and protein 7a can function as redundant separate forces against the eIF2α phosphorylation pathway but both are needed for an optimal antagonistic effect on IFN-α induced antiviral responses.

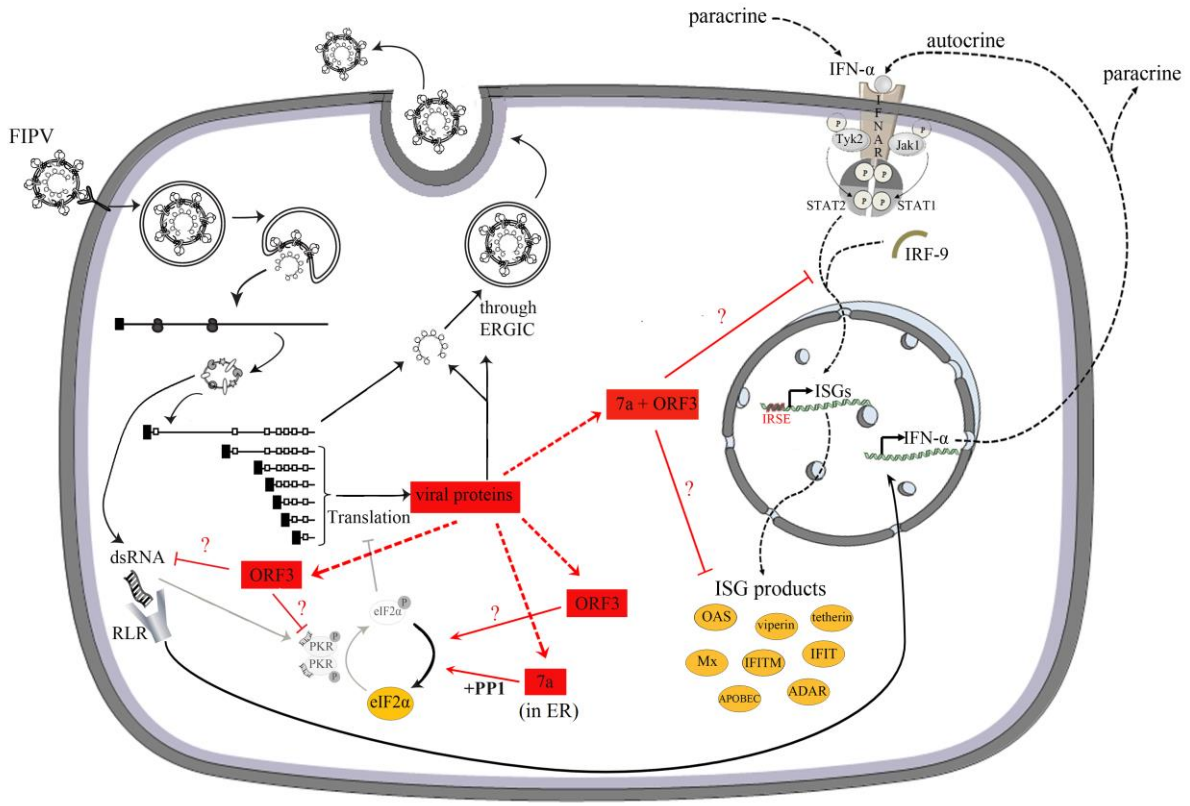

Figure 6.1: Hypothetical model for the IFN-α-antagonistic properties of protein 7a and ORF3 encoded proteins of FIPV, based on the data obtained in this thesis.

The figure is presented as an adaptation of Figure 1.6B [p33], enclosed in the introduction of this thesis.

Our results indicated that protein 7a interferes with phosphorylation of eukaryotic translation initiation factor 2 alpha (eIF2α). It is suggested that it possibly recruits protein phosphatase 1 (PP1) to initiate the dephosphorylation process, in that way reversing the antiviral activity of IFN-induced dsRNA-dependent protein kinase (PKR). Proteins encoded by ORF3 may also impede the eIF2α phosphorylation, however its exact mode of action was not yet defined. Three speculative mode of actions are represented in the figure with question marks. Furthermore, the combined action of protein 7a and one or more ORF3 encoded proteins may be needed to block the overall IFN antiviral effects and to allow more efficient replication of FIPV. Their action mechanism should be verified in the future, but it can be hypothesized that they probably regulate each other to result in either a block on the JAK/STAT pathway or an interference with the antiviral activities of one or more ISG products (also presented with question marks).

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Chapter 7

Summary - Samenvatting

Summary

Feline infectious peritonitis (FIP) is an extremely deadly disease of the felid population, for which the pathogenesis is still not completely elucidated. The virus (FIPV) that causes this disease, encodes for several structural, non-structural and accessory proteins. It is generally claimed that the accessory proteins are important for virus-host interactions, as accessory open reading frames (ORF) deletion mutants of type II FIPV-wt (FIPV-Δacc) are attenuated *in vivo*. Nevertheless, their exact functions are not unravelled, which forms the central aim of this thesis. Identifying the accessory proteins that render FIPV highly virulent, would generate new opportunities to treat this awful disease.

The **first chapter** is divided in three sections. First, an introduction is given about FIPV in general, describing its historical background, taxonomy, virion structure, replication cycle, pathogenesis and immune evasion strategies. The second section consists of the antiviral effects of interferon on positive single stranded RNA viruses, as interferon is of major importance in the current thesis. Finally, the introduction chapter is closed with an overview of the coronavirus countermeasures against interferon-induced responses.

In **Chapter 2**, the aims of this thesis are specified.

In **Chapter 3**, a possible function of FIPV accessory proteins during replication in the primary target cell, the monocyte, was investigated. Sustained and productive replication in monocytes/macrophages is one of the most important differences between low virulent FECV and lethal FIPV. It has already been demonstrated by our research group (Dewerchin et al., 2005) that the replication of FECV in peripheral blood monocytes drops after 12h post inoculation, while FIPV sustains its replication in monocytes from 45% of the cats. In order to examine if the accessory ORFs of FIPV are important for this sustained replication, we first identified three cats out of our household SPF cats that exhibit the sustained infection kinetic pattern for FIPV-wt. Further, the monocytes of these cats were used to establish the infection kinetics for different FIPV-Δacc. Three FIPV-Δacc were used, which were kindly provided by Prof. Dr. Rottier (Faculty of Veterinary Medicine, Utrecht University). They are deleted in ORF3abc, ORF7ab, or both ORF3abc and ORF7ab, and defined as FIPV-Δ3, FIPV-Δ7 and FIPV-Δ3Δ7, respectively. We demonstrated that both FIPV-Δ7 and FIPV-

Δ3Δ7 could no longer sustain their replication in the monocytes after 12hpi, in contrast to FIPV-wt. On the other hand, FIPV-Δ3 infected the monocytes productively up till 24hpi, although its infection was less efficient than that of FIPV-wt. Together, these data represent the importance of accessory ORFs for FIPV replication of monocytes/macrophages, which may aid to explain their preservation and value *in vivo*. Especially, the presence of an intact ORF7 is fundamental to establish a productive infection of the *in vivo* target cell by FIPV, while it appears that ORF3 encoded proteins may have only a supportive role.

In **Chapter 4**, it was further explored which mechanisms could explain the observed relevance of the accessory ORFs during virus-target cell interactions. We focussed on host immunity, as this is one of the important factors that influences disease outcome. Numerous studies demonstrated that the virus is able to evade different facets of the host immune response in order to generate a fatal disease. The humoral immunity does not protect against FIPV infection while the strength of the cell-mediated adaptive immunity determines the outcome of a FIPV infection. Establishing a strong cell-mediated immune response depends on the amount of proliferation and activation of immune cells. This activation largely relies on virus replication, antigen-presentation and cytokine expression (e.g. IFN-γ), being factors that are, in the first place, controlled by the innate immunity through type I IFNs. The speed and efficiency by which a virus can antagonize the innate immunity, may be critical determinants in its pathogenicity. Presently, little is known about the type I IFN-induced innate immunity during FIPV infections. Studying the interplay between FIPV and IFN-α could answer unsolved questions about the FIP pathogenicity. First, our results demonstrated that FIPV infection induced the production of IFN-α, both *in vivo* and *in vitro*. This indicates that FIPV is not able to evade the IFN induction cascade but can still replicate in the presence of IFN-α. Knowing that several coronavirus accessory genes play a central role in counteracting antiviral responses, we examined if the ORF7 encoded proteins of FIPV also have such antagonistic properties. FIPV-Δ7 was more susceptible to IFN-α treatment than FIPV-wt and protein 7a, but not protein 7b, could rescue FIPV-Δ7 from this IFN sensitivity. Nevertheless, our results also indicate that protein 7a is closely collaborating with ORF3 to antagonize the antiviral response. This conclusion was drawn because protein 7a could no longer restore the replication efficiency of FIPV-Δ3Δ7 in the presence of IFN-α and because all three FIPV-Δacc, are equally sensitive to IFN-α treatment.

Since Chapter 4 showed that protein 7a appears to circumvent the IFN-mediated antiviral response, we further delved into its exact mode of action in **Chapter 5**. FIPV protein 7a is closely related to TGEV protein 7, which has previously been identified as an inhibitor of the phosphorylation of the translation initiation factor eIF2α by interaction with the cellular phosphatase PP1. Indeed, eIF2α phosphorylation is one of the antiviral effector mechanisms of IFN-α, resulting in translation inhibition. We found that protein 7a of FIPV potently reduced the eIF2α phosphorylation induced by both a chemical ER stressor (thapsigargin) and infection. Similar to TGEV protein 7, protein 7a of FIPV possesses a PP1c binding motif. Deleting this motif impaired the interference with phosphorylation of eIF2α, indicating that PP1 may be involved in this process. Next to these findings, infection studies with FIPV-Δacc implied that also ORF3 encoded proteins were able to impede this pathway, as only FIPV-Δ3Δ7 was inducing a significant higher eIF2α phosphorylation level than FIPV-wt. The exact mode of action of ORF3 has not been explored in depth during this thesis, but is certainly of interest for further research. In conclusion, both protein 7 and ORF3 encoded protein(s) seem to execute a redundant role in dephosphorylating eIF2α, being one of the countermeasures of FIPV on the IFN antiviral response.

Chapter 6 combines and discusses all the findings of this current thesis. As a general conclusion, it can be stated that the accessory proteins of FIPV attribute to the productive infection of the *in vivo* target cell, the monocytes/macrophages, with a major role for ORF7. Moreover, interfering with the innate immune response, induced by IFN-α, was proposed as an important function for protein 7a and ORF3. Dephosphorylation of eukaryotic translation initiation factor eIF2α, was one of the actions of protein 7a that was characterized in depth, but this could not fully explain its IFN-α antagonistic property. Most likely, a combination of protein 7a and ORF3 encoded proteins is required to efficiently overcome the IFN-α induced antiviral response, eventually allowing more efficient replication in the host cell.

Samenvatting

Feliene infectieuze peritonitis is een extreem dodelijke ziekte in de kattenpopulatie, waarvan de pathogenese nog steeds niet volledig opgehelderd is. Het virus (FIPV) dat deze ziekte veroorzaakt, codeert voor verschillende structurele, niet-structurele en accessoire eiwitten. Het is algemeen aangenomen dat de accessoire eiwitten belangrijk zijn voor virus-gastheer interacties, onder andere omdat de accessoire openleesraam (OLR) deletiemutanten van FIPV (FIPV-Δacc) geheel verzwakt zijn *in vivo*. Hun exacte functies zijn echter nog niet onthuld, en dit vormt de centrale doelstelling van deze thesis. Het identificeren van de accessoire eiwitten die de hoge virulentie van FIPV mee bepalen, zou nieuwe mogelijkheden bieden om deze vreselijke ziekte te behandelen.

Het **eerste hoofdstuk** is onderverdeeld in drie delen. Eerst wordt een algemene introductie over FIPV gegeven, waarin de historische achtergrond, taxonomie, virion structuur, replicatiecyclus, pathogenese en immuun-evasie strategieën worden besproken. Aangezien interferon een belangrijk element vormt in deze thesis, worden in het tweede deel van dit inleidend hoofdstuk de antivirale effecten van interferon op positief enkelstrengige RNA virussen beschreven. Tot slot wordt een overzicht gegeven van de mechanismen die coronavirussen gebruiken om deze interferon geïnduceerde respons te omzeilen.

Hoofdstuk 2 beschrijft de specifieke doelstellingen van deze thesis.

In **hoofdstuk 3** wordt een mogelijke functie van de accessoire eiwitten van FIPV gedurende de replicatie in de primaire doelwitcel, de monocyt, onderzocht. Eén van de belangrijkste verschillen tussen het laag virulente FECV en dodelijke FIPV is namelijk de progressieve en productieve replicatie in monocyt/macrofagen. Eerder werd aangetoond door ons onderzoeksteam (Dewerchin *et al.*, 2005) dat de replicatie van FECV in perifere bloedmonocyten altijd abortief was vanaf 12h na inoculatie, terwijl FIPV zijn replicatie in de monocyten van 45% van de katten kan onderhouden. Om te achterhalen of de accessoire OLR van FIPV hiervoor belangrijk zijn, hebben we eerst drie katten geïdentificeerd welke deze typische progressieve infectiekinetiek voor FIPV-wt vertoonden. Daarna werden de monocyten van deze katten gebruikt om de infectiekinetieken voor verschillende FIPV-Δacc op te stellen. Drie verschillende FIPV-Δacc werden gebruikt (aangeboden door Prof. Dr.

Rottier, Faculteit Diergeneeskunde, Universiteit van Utrecht). Deze mutanten zijn gedeleteerd in OLR3abc, OLR7ab of beide OLRs, en worden respectievelijk aangeduid als FIPV-Δ3, FIPV-Δ7 en FIPV-Δ3Δ7. We toonden aan dat, in tegenstelling tot FIPV-wt, zowel FIPV-Δ7 als FIPV-Δ3Δ7 niet langer hun replicatie in de monocyten productief konden onderhouden tot 24h na inoculatie. FIPV-Δ3 kon dit wel, maar infectie met dit virus was minder efficiënt dan die van FIPV-wt. Dit toont aan dat de accessoire OLRs belangrijk zijn voor FIPV om replicatie in monocyt/macrofagen te onderhouden, wat mede de instandhouding en waarde van deze genen *in vivo* verklaart. Voornamelijk de aanwezigheid van een intact OLR7 lijkt van fundamenteel belang voor progressieve infectie van de *in vivo* doelwitcel van FIPV, terwijl OLR3 eerder een ondersteunende rol lijkt te spelen.

In **hoofdstuk 4** hebben we de mechanismen verder onderzocht die de geobserveerde relevantie van de accessoire OLRs gedurende virus-doelwitcel interacties kunnen verklaren. We hebben ons geconcentreerd op de gastheer immuniteit, omdat dit één van de factoren is die het ziekteverloop en de afloop bepalen. Verschillende studies hebben aangetoond dat het virus verschillende aspecten van de immuunrespons van de gastheer kan ontlopen om zo een fatale ziekte te veroorzaken. Terwijl het humorale immuunsysteem geen bescherming biedt, lijkt afloop van een FIPV infectie bepaald te worden door de sterkte van de cel-gemedieerde aangepaste immuniteit. De proliferatie en activatie capaciteit van immuuncellen bepalen hierbij of een sterke cel-gemedieerde immuunrespons kan bewerkstelligd worden. Deze activatie is grotendeels afhankelijk van virus replicatie, antigeenpresentatie en cytokine expressie (zoals IFN-γ), welke factoren zijn die in de eerste plaats gecontroleerd worden door de aangeboren immuniteit via type I IFNs. Kritische factoren voor de pathogeniciteit van een virus zijn de snelheid en efficiëntie waarmee het deze aangeboren immuniteit kan tegenwerken. Tot op heden is weinig gekend over de type I IFN-geinduceerde aangeboren immuunrespons tijdens een FIPV infectie. Dit bestuderen zou onopgeloste vragen over FIPV pathogeniciteit kunnen beantwoorden. Onze resultaten toonden aan dat FIPV infectie wel degelijk de productie van IFN-α induceert, en dit zowel *in vitro* als *in vivo*. Dit geeft aan dat, ondanks het feit dat FIPV de IFN productie cascade niet kan ontlopen, het wel in de aanwezigheid van IFN-α kan repliceren. Wetende dat verschillende coronavirus accessoire genen een centrale rol spelen in de tegenwerking van antivirale responsen, hebben wij dan ook onderzocht of ORF7 van FIPV ook antagonistische eigenschappen tegenover FIPVvertoont. FIPV-Δ7 was gevoeliger voor IFN-α behandeling dan FIPV-wt en eiwit 7a, maar niet eiwit 7b, kon de gevoeligheid van FIPV-Δ7 opheffen. Desalniettemin toonden onze

resultaten ook aan dat een dichte samenwerking tussen eiwit 7a en ORF3 nodig was om de antivirale respons te ontwijken. Deze conclusie kon gemaakt worden omdat, in aanwezigheid van IFN-α , eiwit 7a niet langer de replicatie efficiëntie van FIPV-Δ3Δ7 kon verhogen en omdat alle drie FIPV-Δacc een gelijkaardig negatief effect van IFN-α behandeling ondervonden.

Aangezien hoofdstuk 4 leek aan te tonen dat eiwit 7a de IFN-gemedieerde antivirale respons gedeeltelijk kon tegengaan, hebben we verder uitgezocht in **hoofdstuk 5** wat het exact werkingsmechanisme van dit eiwit kon zijn. Eiwit 7a van FIPV is sterk homoloog aan eiwit 7 van TGEV. Deze laatste werd eerder geïdentificeerd als een inhibitor van de fosforylatie van translatie initiatie factor eIF2α via zijn interactie met het cellulair fosfatase PP1. EIF2α fosforylatie is één van de antivirale effector mechanismen van IFN-α, welke resulteert in translatie inhibitie. Wij vonden dat ook eiwit 7a van FIPV de eIF2α fosforylatie, geïnduceerd door zowel een chemische ER stressor (thapsigargin) als infectie, kon reduceren. Eiwit 7a van FIPV bevat, net zoals eiwit 7 van TGEV, een PP1c bindingsmotief. Wanneer dit motief gedeleteerd werd kon eiwit 7a niet langer de eIF2α fosforylatie tegengaan, wat aangeeft dat PP1 zou kunnen betrokken zijn in dit proces. Naast deze bevindingen, toonden infectiestudies met FIPV-Δacc aan dat eiwitten gecodeerd door OLR3 ook met deze pathway konden interfereren, aangezien enkel FIPV-Δ3Δ7 een significant sterkere eIF2α fosforylatie dan FIPV-wt induceerde. Het exacte werkingsmechanisme van OLR3 werd niet verder uitgezocht in deze thesis, maar is zeker interessant om verder te bestuderen. Er kan besloten worden dat zowel eiwit 7a als eiwitten gecodeerd door OLR3 een complementaire rol vervullen in de defosforylatie van eIF2α, wat één van de IFN-evasie mechanismen is van FIPV.

Hoofdstuk 6 combineert en bediscussieert alle bevindingen van deze thesis. Algemeen kan er besloten worden dat de accessoire eiwitten van FIPV bijdragen tot de productieve infectie van de *in vivo* doelwitcel, de monocyt/macrofaag, met een hoofdrol voor OLR7. Bovendien werd aangenomen dat eiwit 7a en OLR3 samen kunnen interfereren met het aangeboren immuunsysteem geïnduceerd door IFN-α. Eén van de werkingen van eiwit 7a waarop dieper werd ingegaan was de defosforylatie van eIF2α, maar dit kon wel niet volledig zijn IFN-α antagonistische eigenschap verklaren. Hoogstwaarschijnlijk is een combinatie van eiwit 7a en één of meerdere eiwitten gecodeerd door OLR3 nodig om de IFN-α geïnduceerde antivirale respons efficiënt te omzeilen en zo meer efficiënte replicatie in de gastheercel toe te laten.

Curriculum Vitae

Personalia

Annelike Dedeurwaerder werd geboren op 16 januari 1986 te Kortrijk. Zij beëindigde haar secundaire studies in 2004 aan het Onze-Lieve-Vrouw-instituut van Vlaanderen te Kortrijk (richting Wetenschappen-Wiskunde). Vervolgens startte zij haar universitaire studies aan de Faculteit Bio-ingenieurswetenschappen van de Universiteit Gent waar zij in 2009 het diploma behaalde van Master in de Bio-ingenieurswetenschappen afstudeerrichting cel- en genbiotechnologie met onderscheiding. Sinds augustus 2009 verricht zij onderzoek aan de Vakgroep Virologie, Parasitologie en Immunologie op de Faculteit Diergeneeskunde van de Universiteit Gent. Vanaf januari 2010 beschikt zij over een vierjarig IWT-doctoraatsbursaal van het Vlaamse Agentschap voor Innovatie door Wetenschap en Techniek. Dit onderzoek handelt over het feliene infectieuze peritonitis virus en de rol van zijn accessoire eiwitten in het bereiken van een hoge virulentie door onder andere de evasie van de interferon-gemedieerde immuunrespons.

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