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The sensitivity of Parainfluenza virus 5 to IFIT proteins

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**A thesis submitted to St George's, University of London
for the degree of Doctor of Philosophy**

Declaration

All of the work presented in this thesis is my own unless stated otherwise.



Jack Hankinson

Abstract

Parainfluenza virus 5 (PIV5) is a negative sense RNA virus of the *Paramyxoviridae* family which causes both acute and persistent infections in cell culture and in vivo. Previous work had implicated alterations in the P protein which affect viral polymerase activity, as well as sensitivity to the interferon (IFN) system, as mechanisms by which persistent infection may be established. In the current study, the impact of alterations in the P protein on viral RNA synthesis were investigated by using a viral minigenome reporter assay, and changes around sites of phosphorylation in the P protein were found to have a dramatic effect on the rate of viral transcription, which we propose as a potential 'switch' mechanism between acute and persistent infections. Our previous work had identified IFIT1, which inhibits the translation of viral mRNA, as the IFN-stimulated gene primarily responsible for the sensitivity of PIV5 to IFN. Here, using minigenome reporter assays, PIV5 was shown to be sensitive to multiple IFIT proteins, in contrast to Measles virus which was insensitive. The sensitivity of PIV5 to IFIT1 was found to be due its failure to generate a fully-methylated mRNA cap structure, as determined by mass spectrometry analysis. In the course of our previous work on IFIT1, we showed that the effect of IFIT1 on translation was not equal between PIV5 genes. By demonstrating that cap-proximal RNA secondary structure affects the affinity of IFIT1 for viral mRNA, the differential sensitivity of PIV5 genes to IFIT1 was here shown to be a function of the presence or absence of RNA hairpin structures in the 5' untranslated regions (5'UTRs) of PIV5 transcripts. The introduction of RNA secondary structure into an otherwise unstructured PIV5 5'UTR can confer protection from IFIT1 and reduce the inhibition of PIV5 growth by IFN. The implications of P mutations and gene-specific sensitivity to IFIT1 are discussed in terms of persistent infection.

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Abbreviations

APMV6	Avian paramyxovirus 6	MPRPV	Mapuera virus
+ssRNA	Positive sense, single-stranded RNA	MTase	Methyltransferase
BiMG	Bicistronic minigenome	MuV	Mumps virus
bp	Base pairs	MV	Measles virus
BSA	Bovine serum albumin	NDV	Newcastle disease virus
CAPAM	Cap-Specific Adenosine Methyltransferase	NiV	Nipah virus
CAT	Chloramphenicol acetyltransferase	NP	Nucleoprotein
CDS	Coding sequence	nt	Nucleotide
CDV	Canine distemper virus	P	Phosphoprotein
CMTR1/2	Cap Methyltransferase 1/2	PAMP	Pathogen-associated molecular pattern
CPE	Cytopathic effect	PCIF1	Phosphorylated CTD Interacting Factor 1
DI	Defective interfering	pfu	Plaque-forming unit
DMSO	Dimethyl sulphoxide	PIV5	Parainfluenza virus 5
dpi	Days post-infection	PNK	Polynucleotide kinase
DVG	Defective viral genome	PORPV	Porcine rubulavirus
EBOV	Ebola virus	PPIV1	Porcine parainfluenza 1
EMSA	Electrophoretic mobility shift assay	PRR	Pattern recognition receptor
F	Fusion protein	PTM	Post-translational modification
FBS	Foetal bovine serum	RABV	Rabies virus
HeV	Hendra virus	RIG-I	Retinoic acid-inducible gene I
HN	Haemagglutinin-neuraminidase	RPP	RNA 5'-pyrophosphatase
hpi	Hours post-infection	RPV	Rinderpest virus
HPIV2	Human parainfluenza virus 2	RRL	Rabbit reticulocyte lysate
HPIV3	Human parainfluenza virus 3	rSAP	Shrimp alkaline phosphatase
HPIV4A	Human parainfluenza virus 4a	SDS	Sodium dodecyl sulphate
IFIT	IFN-induced protein with tetratricopeptide repeats	SDS-PAGE	SDS-polyacrylamide gel electrophoresis
IFN	Interferon	SeV	Sendai virus
IGS	Intergenic sequence	SH	Short hydrophobic protein
IRF	Interferon regulatory factor	SNP	Single nucleotide polymorphism
ISG	Interferon-stimulated gene	-ssRNA	Negative sense, single-stranded RNA
ISRE	Interferon-stimulated response element	SV41	Simian virus 41
IVT	In vitro transcription	T7-IVT	T7 in vitro transcription
L	Large polymerase protein	TCID50	Tissue culture infection dose 50%
LGP2	Laboratory of genetics and physiology 2	UTR	Untranslated region
M	Matrix protein	VCE	Vaccinia capping enzyme
MARV	Marburg virus	VEEV	Venezuelan equine encephalitis virus
MDA5	Melanoma differentiation-associated protein 5	VSV	Vesicular stomatitis virus
MG	Minigenome	XRN1	5'-3' exoribonuclease 1
moi	Multiplicity of infection	ZAP-L/S	Zinc-finger antiviral protein Long/Short

Chapter 1 – Introduction

This project will investigate several aspects of the biology of Parainfluenza virus 5 (PIV5), a member of the *Paramyxoviridae* family. This chapter introduces the taxonomy and life cycle of these viruses, the mechanisms by which they replicate, and how they interact with the host innate immune response (the interferon system). The aims of the project are stated at the end of the chapter.

1.1 Paramyxoviruses

Paramyxoviruses are negative-sense, single-stranded RNA (-ssRNA) viruses within the family *Paramyxoviridae* and include important human pathogens such as Measles virus, Mumps virus, and Nipah virus¹. The *Paramyxoviridae* family is classified within the *Mononegavirales* order, which also includes the *Filoviridae* (e.g. Ebola virus), *Rhabdoviridae* (e.g. Rabies virus and Vesicular stomatitis virus), and *Pneumoviridae* (e.g. Respiratory syncytial virus) families among others². Paramyxoviruses share conserved genomes and replication strategies across multiple mammalian, avian, reptilian, and marine hosts. The wide range of hosts and the prevalence of these viruses makes them especially relevant in terms of possible emerging diseases and ongoing morbidity caused by endemic diseases.

1.1.1 Taxonomy and general virology

As of 2019, within *Paramyxoviridae* there are four sub-families: *Avulavirinae*, *Metaparamyxovirinae*, *Orthoparamyxovirinae*, and *Rubulavirinae*¹. The genera and the individual viruses are summarised in figure 1.1. The former *Avulavirus* and *Rubulavirus* genera have recently been reclassified but the old nomenclature will still be used in this thesis; all members of the *Avulavirinae* and *Rubulavirinae* subfamilies will be referred to simply as avulaviruses and rubulaviruses, respectively.

<i>Avulavirinae</i>		<i>Rubulavirinae</i>			
<i>Orthoavulavirus</i>		<i>Orthorubulavirus</i>		<i>Pararubulavirus</i>	
Newcastle Disease virus	NDV / APMV1	Human parainfluenza virus 2	HPIV2	Achimota virus 1	AchiV1
Avian paramyxovirus 9	APMV9	Human parainfluenza virus 4	HPIV4	Achimota virus 2	AchiV2
Avian paramyxovirus 12	APMV12	Parainfluenza virus 5	PIV5	Menangle virus	MenV
Avian paramyxovirus 13	APMV13	Mapuera virus	MapV	Sosuga virus	SoRV
Avian paramyxovirus 16	APMV16	Mumps virus	MuV	Teviot virus	TeV
Antarctic penguin virus A	APV-A	Porcine rubulavirus	PRV	Tioman virus	TioV
Antarctic penguin virus B	APV-B	Simian virus 41	SV41	Tuhoku virus 1	TuV1
Antarctic penguin virus C	APV-C			Tuhoku virus 2	TuV2
				Tuhoku virus 3	TuV3

<i>Orthoparamyxovirinae</i>					
<i>Henipavirus</i>		<i>Morbillivirus</i>		<i>Respirovirus</i>	
Cedar virus	CedV	Canine distemper virus	CDV	Bovine parainfluenza virus 3	BPIV3
Ghana virus	GhV	Dolphin morbillivirus	DMV	Caprine parainfluenza virus 3	CPIV3
Hendra virus	HeV	Feline morbillivirus	FeMV	Human parainfluenza virus 1	HPIV1
Mojang virus	MojV	Measles virus	MV	Human parainfluenza virus 3	HPIV3
Nipah virus	NiV	Phocine distemper virus	PDV	Sendai virus	SeV
		Rinderpest virus	RPV	Porcine parainfluenza virus 1	PPIV1
		Peste des petits ruminants virus	PPRV		

Figure 1.1 - Paramyxoviridae taxonomy. An incomplete selection of genera are shown which contain the most well-studied and important paramyxoviruses. The genera are divided into the subfamilies Avulavirinae, Rubulavirinae, and Orthoparamyxovirinae.

Paramyxovirus genome size varies from 14.5 kilobases (kb) to 20.1 kb¹ and all paramyxoviruses have the same virion structure (figure 1.2A) and genome layout with some minor variations (figure 1.2B). From 3' to 5' of the -ssRNA genome, paramyxoviruses minimally encode the following: a nucleoprotein (NP or N) which encapsidates the genome and recruits the viral RNA-dependent RNA polymerase (RdRp); a phosphoprotein (P) which is a cofactor of the RdRp and mediates interactions between viral proteins; a matrix protein (M) which interacts with NP and the viral glycoproteins to promote assembly and release; a fusion protein (F) which mediates fusion between the virion and cell membranes to facilitate viral entry; an attachment protein (H, HN, or G) which attaches to cell surface receptors prior to fusion; and a large polymerase protein (L) which performs multiple enzymatic activities and, in complex with P, is responsible for viral genome replication and transcription.

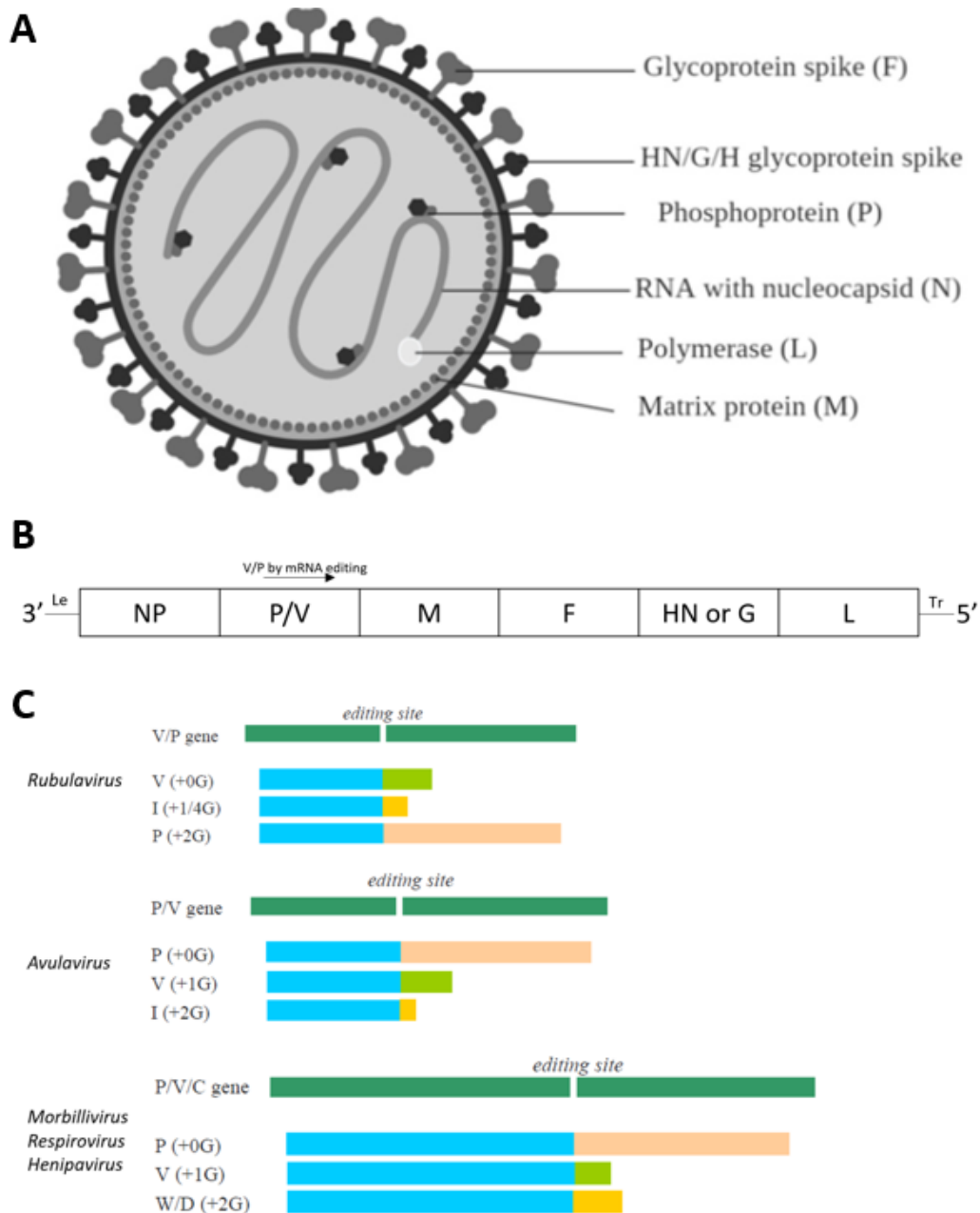


Figure 1.2 - The conserved virion structure and genome layout of paramyxoviruses. A) The structure of a paramyxovirus virion. **B)** The minimal set of ORFs encoded by all paramyxoviruses are shown by their position in the negative sense viral genome: NP, nucleoprotein; P, phosphoprotein; M, matrix protein; F, fusion protein; HN or G; haemagglutinin/neuraminidase or glycoprotein; L, large polymerase protein. Also shown are the regulatory Leader (Le) and Trailer (Tr) sequences. The P ORF overlaps with an ORF encoding V and/or C (not shown), as detailed in C. Some viruses additionally encode a short hydrophobic (SH) protein between F and HN/G (not shown). **C)** mRNA editing of the various paramyxovirus genera. Either the P or V protein is generated by a by mRNA editing of the genomic ORF which results in the insertion of non-templated guanosines and a shift of the reading frame. The I protein of rubulaviruses and avulaviruses is a minor product and is not known to have any function. Figure A adapted from Wikipedia Commons: <https://commons.wikimedia.org/wiki/File:Ijms-20-04657-g007.webp>.

In addition to the proteins listed above, paramyxoviruses encode one or more accessory proteins within the P open reading frame (ORF). For *Orthoparamyxovirinae*, these include the C protein produced by leaky scanning during transcription, and the V protein produced by mRNA editing of the P transcript. mRNA editing involves the co-transcriptional insertion of non-

templated guanosines by the viral RdRp at a specific sequence which shifts the reading frame³⁻⁵. The various mRNA editing mechanisms of paramyxovirus genera are shown in figure 1.2C. In contrast to most other paramyxovirus genera, rubulaviruses encode a V protein in the genomic ORF and produce the full-length P protein by mRNA editing of the V transcript. The primary function of paramyxovirus accessory proteins is evasion of the innate immune system, typically by either preventing recognition of viral RNA by cellular receptors⁶ or disrupting JAK-STAT signalling⁷⁻⁹. The V protein may have further roles in regulation of RNA synthesis¹⁰⁻¹⁴ and other viral processes, the absence of which result in attenuation of the virus when V is deleted from the genome¹⁵⁻¹⁷. Additionally, some rubulaviruses encode a short hydrophobic membrane protein (SH) which is non-essential but has a possible role in avoiding the induction of apoptosis^{18,19}.

Paramyxovirus virions are enveloped and pleiomorphic with a diameter in the range of 300-500nm¹. In the virion structure, the HN/G and F proteins are embedded in the viral membrane, and the M protein is interior to this and interacts with the cytoplasmic domains of the glycoproteins and NP, which encapsidates the RNA genome and is bound to multiple copies of the L/P viral polymerase. The V protein is also incorporated into virions⁸, though is not shown in figure 1.2A.

1.1.2 The paramyxovirus replication cycle

A diagram of the life cycle of paramyxoviruses is shown in figure 1.3. Attachment of the virion to the host cell is dependent upon the binding of the viral attachment protein (H/HN/G) to the host cell receptor, which varies between viruses. Viruses including PIV5, NDV, and HPIV3 encode HN surface proteins which have neuraminidase activity and utilise ubiquitous sialic acids for attachment to the host cell²⁰⁻²². Other paramyxoviruses use more specific cell receptors, for example Nipah virus which uses Ephrin-B2²³ and Measles virus which uses SLAM/CD150²⁴.

Following attachment to the host cell receptor, membrane fusion is mediated by the viral F protein and the paramyxovirus ribonucleocapsid (RNP) is deposited in the cytoplasm of the cell. The RNP consists of the -ssRNA genome encapsidated by NP so that each NP monomer binds 6 nucleotides of the genome^{25,26}. This restricts genome length to multiples of 6 to ensure full coverage by NP – a requirement known as the ‘rule of six’^{27,28}. Full encapsidation by NP avoids innate immune sensing, enables packaging of nascent genomes into virions^{29,30}, and regulates the switch between viral transcription and replication³¹⁻³³.

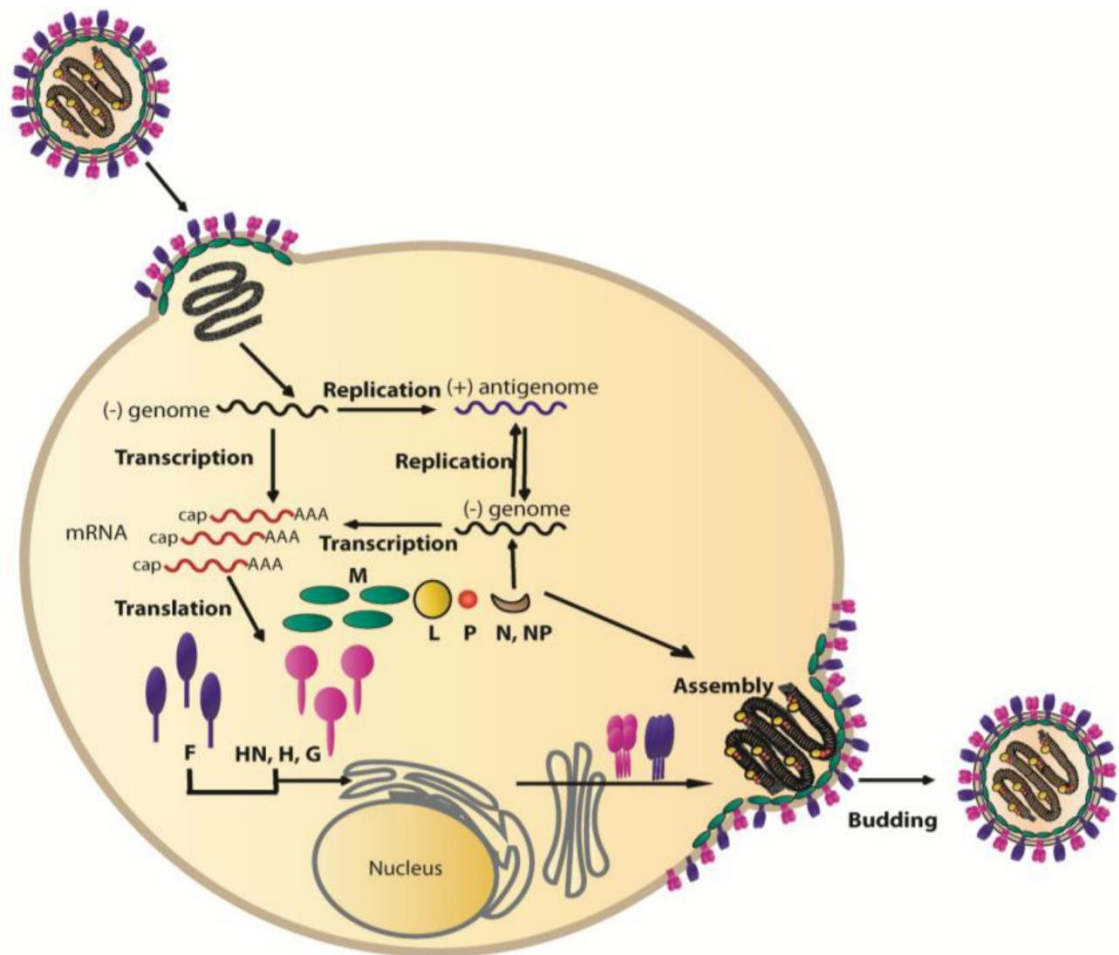


Figure 1.3 – The paramyxovirus life cycle. The individual steps of entry, transcription, replication, assembly, and budding are described in the main text. Figure adapted from Najjar et al. 2014³⁴.

The L and P proteins are bound to the incoming RNP and form the viral RNA-dependent RNA polymerase (RdRp). The P protein interacts with both the L protein^{35,36} and the NP protein³⁷ to mediate assembly of replication complexes, and its activity is regulated by numerous phosphorylations and other post-translational modifications^{38–40} (figure 1.4). The L protein consists of multiple domains and is responsible for several enzymatic activities^{41–43}: RNA-dependent RNA polymerase, mRNA cap guanylyltransferase, mRNA cap guanine-N7-methyltransferase, and mRNA cap 2'-O-methyltransferase. Viral transcription is initiated upon infection when the RdRp is recruited to the bipartite leader promoter sequence (Le) at the 3' end of the encapsidated genome^{44,45}. Following transcription of the NP gene, polyadenylation of the transcript occurs by stuttering of the polymerase on a short polyA tract^{46,47}. Genes are separated by intergenic sequences (IGSs) which vary in length and sequence between viruses and individual genes⁴⁸. At IGSs, the RdRp either remains bound to the viral genome and proceeds to scan for the next gene start signal before transcribing the next gene, or it dissociates from the genome at a certain frequency which is unknown but possibly regulated by the sequence of the

IGS^{33,49}. Having dissociated, the RdRp can only bind the genome again at terminal Le sequence; this results in greater accumulation of the products of 3'-proximal genes than those of 5'-proximal genes in the viral genome⁵⁰. Therefore, the order of genes (5'-NP-P/V-M-F-HN-L-3') reflects the relative amount of each gene product produced during an infection.

As shown in figure 1.4, the P protein mediates interactions between the L polymerase and the NP-encapsidated genome and is essential for viral RNA synthesis. The P protein bears many post-translational modifications, especially phosphorylations, which may alter its function in regulating viral RNA synthesis (see section 1.1.4 on PIV5 for more information).

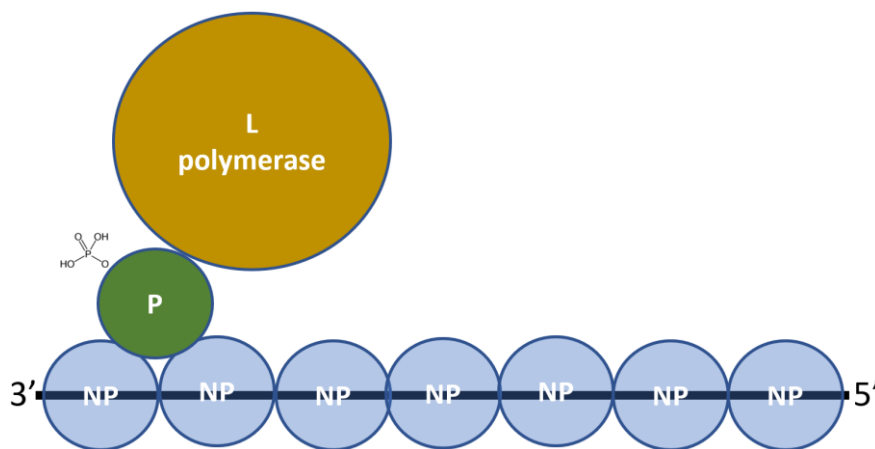


Figure 1.4 - A schematic of the paramyxovirus RNP. The single-stranded negative sense RNA genome (black, not to scale) is encapsidated by the nucleocapsid protein (NP, blue) so that each NP monomer interacts with precisely 6 nucleotides in a sequence-independent manner. The viral phosphoprotein (P, green) interacts with both NP and L, the viral RdRp, to mediate formation of the replication complex. The P protein is subject to multiple post-translational modifications, especially phosphorylations, which may regulate its function.

As the infections proceeds, the primary form of RNA synthesis shifts from transcription to replication of the full-length genome. This process is not fully understood but is likely regulated by the local concentration of NP³¹⁻³³. According to this model, once the amount of NP reaches a level sufficient to encapsidate nascent RNA, the RdRp switches to a 'super-processive' mode, ignores gene start and stop signals, and synthesises a full length copy of the genome^{31,51,52}; this is termed the antigenome and is positive sense (although not translated). The RdRp is recruited to the 3' end of the antigenome by the trailer promoter sequence (Tr) and synthesises new copies of the negative sense viral genome. Tr is a stronger promoter than Le and thus the viral genome accumulates to a higher level than the antigenome.

Nascent encapsidated genomes are packaged into new virions along with copies of P and L. The matrix protein mediates RNP packaging at areas of the cell membrane expressing the viral membrane proteins^{29,30}, and leads to budding of nascent virions. Virus-like particles (particles

lacking the viral genome) can be produced by overexpression of paramyxovirus M proteins, either alone^{53–55} or in combination with NP and F or HN^{29,56,57}, demonstrating the importance of M in the formation of viral particles. Paramyxoviruses can spread to new cells either by the production of infectious particles, or by formation of syncytia (multinucleate cells) by cell-cell fusion mediated by the F protein⁵⁸.

1.1.3 Diseases caused by paramyxoviruses

Many important human pathogens are found in the *Paramyxoviridae* family. The *Morbillivirus* genus includes Measles virus (MV), which in areas of low vaccine coverage can cause considerable morbidity and occasional neurological sequelae⁵⁹. The *Henipavirus* genus includes Nipah virus (NiV) and Hendra virus (HeV), both zoonotic infections (bats are the main reservoir) with high mortality rates in humans which are considered high-risk candidates for emerging epidemics^{60,61}. The *Rubulavirus* genus includes Mumps virus (MuV), another vaccine-preventable disease that mostly causes mild symptoms but with occasional serious sequelae⁶². Newcastle disease virus (NDV, alternatively Avian paramyxovirus 1) infects chickens and can cause large economic losses. There are many paramyxoviruses which have bats as their natural host^{63–65}, which may be of particular importance given the possible reservoir role of bats in the emergence of zoonotic viruses⁶⁶.

1.1.4 Parainfluenza virus 5 (PIV5) and persistent infection

Parainfluenza virus 5 (PIV5, previously known as Simian virus 5 (SV5)) was first isolated from primate cells in the 1950s^{67,68}. It is currently classed as a member of the *Orthorubulavirus* genus within the *Rubulavirinae* subfamily, with the species name *Mammalian orthorubulavirus 5*¹. PIV5 has been isolated from multiple mammalian species but is thought to primarily be a virus of humans⁶⁹, albeit one which causes no known disease in its natural host. PIV5 infection of dogs, however, causes the vaccine-preventable illness kennel cough^{70,71}. The ‘wild-type’ reference strain of PIV5 – W3A – is actually a lab-adapted strain which causes minimal cytopathic effect (CPE) in cell culture⁶⁸.

The genome of PIV5 is 15246 nt and encodes the minimal set of paramyxovirus genes shown in fig 1.2. The second ORF of PIV5 encodes the V protein which shares an N-terminus with the P

protein. The longer P transcript is produced by mRNA editing in which 2 non-templated guanosine nucleotides are inserted, resulting in a switch of ORF from V to full-length P⁵.

Despite not being known to cause any disease in humans, PIV5 serves as a useful model organism for the investigation of both paramyxovirus and *Mononegavirales* biology, in particular the evasion of the host innate immune response. The PIV5 V protein is able to bind to and inhibit the function of cellular RNA-binding receptors⁶, and can target components of innate immune signalling pathways for degradation⁷ (see section 1.2.3).

More recently, the ability of PIV5 to chronically infect cells with minimal cytopathic effect, both in cell culture^{72,73} and in vivo^{74–76}, has raised the possibility of it serving as a model of persistent infection. Persistent infection by RNA viruses is a neglected phenomenon and is likely more common than is currently appreciated⁷⁷. The most prominent examples are chronic infections by hepatitis C virus (HCV) and human immunodeficiency virus 1 (HIV-1) which avoid clearance from the host by generating extensive antigenic diversity to evade the adaptive immune response^{78–80}. However, many other RNA viruses are able to cause persistent infections and, in contrast to HCV and HIV-1, are often apparently asymptomatic.

Despite numerous examples of RNA viruses persisting in vivo, the mechanisms by which this is achieved are not well understood. Investigating how PIV5 can replicate in cell culture without causing significant CPE, and how it can persist in vivo without being cleared by the adaptive immune response, may serve to delineate paradigms of RNA virus persistence which could apply to other paramyxoviruses or *Mononegavirales* members.

Notably, variation at putative sites of phosphorylation in the P proteins of PIV5 strains has been shown to be linked with differing rates of viral RNA synthesis. In particular, the serine at position 157 of the P protein of the lab-adapted W3A strain of PIV5 is phosphorylated and the absence of this serine in some strains (for example F157 in the CPI+ strain of PIV5⁸¹) has previously been shown to increase the rate of viral RNA synthesis^{38,40}. CPI+ was isolated from a dog displaying clinical signs of ‘kennel cough’ and neurological dysfunction^{70,71}, and the potentially increased rate of RNA synthesis of strains such as CPI+ relative to W3A may increase the chances of them being isolated from animals. Many differences exist between the P proteins of PIV5 strains both at phosphorylation sites and their surrounding residues⁶⁹, but the impact of these on viral RNA synthesis and the relative fitness of PIV5 strains remains to be fully determined. The link between P protein variants and PIV5 strain phenotype may be relevant to how PIV5 is able to form persistent infections.

Interferon (see section 1.2) has also been tentatively linked with the persistence of PIV5. Experimental infection of dogs with the CPI+ strain of PIV5 led to a persistently-infected

individual from which virus was isolated from the cerebrospinal fluid⁸². The isolated strain was named CPI- and displays an altered phenotype relative to the parental CPI+ strain: it causes less CPE in cell culture⁸³, is attenuated in vivo⁷¹, and is unable to block interferon signalling⁸⁴ (see section 1.2.3). The counter-intuitive loss-of-function apparently selected for in CPI- has led to the suggestion that increased sensitivity to the host interferon response may promote the formation of persistent infections by PIV5^{77,81,84}. However, it is not clear whether increased sensitivity to interferon is causally linked to persistence and the potential mechanism responsible has not been demonstrated.

Therefore, this project aims to investigate two potential mechanisms which might facilitate persistent infection by PIV5: mutations in the viral polymerase subunit P protein (see figure 1.4) which may regulate RNA synthesis, and the potential for sensitivity to interferon (see section 1.2) to maintain the virus in a quiescent state.

1.2 The interferon (IFN) system

The interferon (IFN) system is the primary host innate response to viral infections^{85,86}. Host cells are able to detect viral nucleic acids and in response produce the cytokine IFN, which is secreted by infected cells and acts in an autocrine and paracrine manner to upregulate hundreds of IFN-stimulated genes (ISGs) involved in the antiviral response. The induced expression of ISGs creates an 'antiviral state' in which viral replication is restricted (figure 1.5). The IFN system is active against multiple types of pathogen, but this section will focus on the IFN system with regard to cytoplasmic RNA viruses.

Mammals encode multiple IFN proteins which are classed in three groups: type I, type II, or type III. The first to be discovered and the most-studied are the type I IFNs, which in humans comprise 13 IFN- α genes, 1 IFN- β gene, and several other genes including IFN- κ and IFN- ω , all on chromosome 9⁸⁶. IFN- α and IFN- β are produced by cells in response to infection by viruses⁸⁷ and their antiviral function is well characterised as being mediated via the interferon- α/β receptor (IFNAR) which is expressed on the surface of most cells. The functions of other type I IFNs are not fully known.

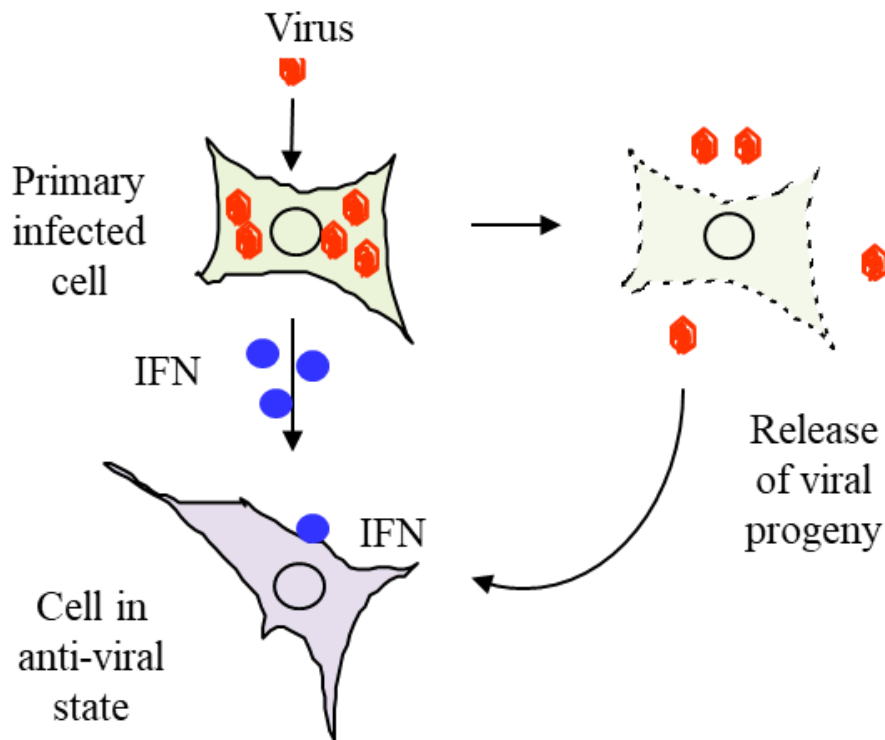


Figure 1.5 - The IFN-induced antiviral state. Cells infected with a virus (red) detect the presence of viral nucleic acids and secrete interferon (IFN, blue). IFN acts autocrinally and paracrinally to induce the expression of IFN-stimulated genes (ISGs) which restrict viral replication and create an 'antiviral state'.

The only type II IFN is IFN- γ ⁸⁸, which is secreted by activated T cells and NK cells⁸⁹ but is not directly induced by viral infection. IFN- γ binds to the interferon-gamma receptor (IFNGR) and regulates the activity of immune cells such as macrophages and B cells.

Type III IFNs comprise IL28A, IL28B, IL29 (also called IFN- λ 2, IFN- λ 3, and IFN- λ 1)^{90,91} and the more recently discovered IFN- λ 4⁹². Type III IFNs are induced by viral infections in a similar manner to type I IFNs⁹³ but unlike IFNAR, expression of the receptors for type III IFNs is mostly restricted to epithelial cells^{90,94}. The functions of type III IFNs are similar to those of type I IFNs and they overlap in the genes they induce⁹⁵, however transcriptomic profiling has revealed type III IFNs to be much less potent^{96,97}.

1.2.1 PAMPs and PRRs

Structures uniquely associated with an infecting virus are called pathogen-associated molecular patterns (PAMPs). PAMPs are not normally found in uninfected cells and include dsRNA, 5' triphosphorylated RNA (ppp-RNA), and cytosolic DNA. To avoid detection by the host cell, many viruses either limit production of PAMPs, use viral proteins to mask them, or antagonise cellular

receptors. A key concept in the detection of PAMPs is the contrast between 'self' structures, which are present in host cells, and 'non-self' structures, which are produced only during viral infections.

Pattern recognition receptors (PRRs) are the host proteins responsible for the detection of PAMPs. These can be found in multiple locations including in the cytosol, in endosomes, and on the cell surface. The PRRs of most relevance to cytoplasmic RNA viruses are the RIG-I-like receptors (RLRs) RIG-I, MDA5, and LGP2. Activation of RIG-I and MDA5 leads to induction of IFN by a common signalling pathway involving MAVS and IRF3; LGP2 cannot interact with MAVS and instead functions as a regulator of RIG-I and MDA5^{98–100}. The primary ligand of RIG-I has been shown to be 5'ppp RNA that forms a hairpin structure so that the 5'-terminal base is paired¹⁰¹. The ligands of MDA5 are more obscure but are likely to include long dsRNA molecules¹⁰².

The binding of RIG-I and MDA5 to their RNA ligands causes a conformational change which exposes the N-terminal CARD domains of the proteins. The CARD domains are then free to interact with the MAVS adaptor protein^{103–105} which serves as a scaffold for the recruitment of multiple signalling components such as TRAF3, TANK, and TBK-1, the latter of which phosphorylates IRF3¹⁰⁶ (and IRF7 when present), resulting in its translocation to the nucleus^{107,108}. Nuclear IRF3 in association with p300-CBP binds to the IFN- β promoter and induces the expression of IFN- β , which is secreted from the infected cell. Although IRF7 expression is very low in most tissues, it is inducible by IFN and can also bind to the IFN- β promoter¹⁰⁹; this has led to the suggestion that IRF7 forms part of a positive feedback mechanism to enhance IFN production^{110,111}.

A parallel signalling pathway operates downstream of MAVS which results in the activation of NF- κ B. As for the IRF3 pathway, MAVS serves as a scaffold for the assembly of a protein complex including TRAF6, NEMO, and IKK β among others. This leads to the phosphorylation of the inhibitor of NF- κ B protein (I κ B), causing its ubiquitination and degradation. No longer bound by I κ B, NF- κ B is translocated to the nucleus¹¹². Binding of NF- κ B to the IFN- β promoter enhances the activation of IRF3-dependent gene expression. The binding of the c-Jun/ATF-2 heterodimer to the promoter also serves to promote the assembly of the entire transcription machinery¹¹³. However, only IRF3 is considered essential because induction of IFN can still occur in the absence of activation of either c-Jun/ATF-2 or NF- κ B^{114,115}.

A diagram of RLR-dependent induction of IFN is shown in figure 1.6.

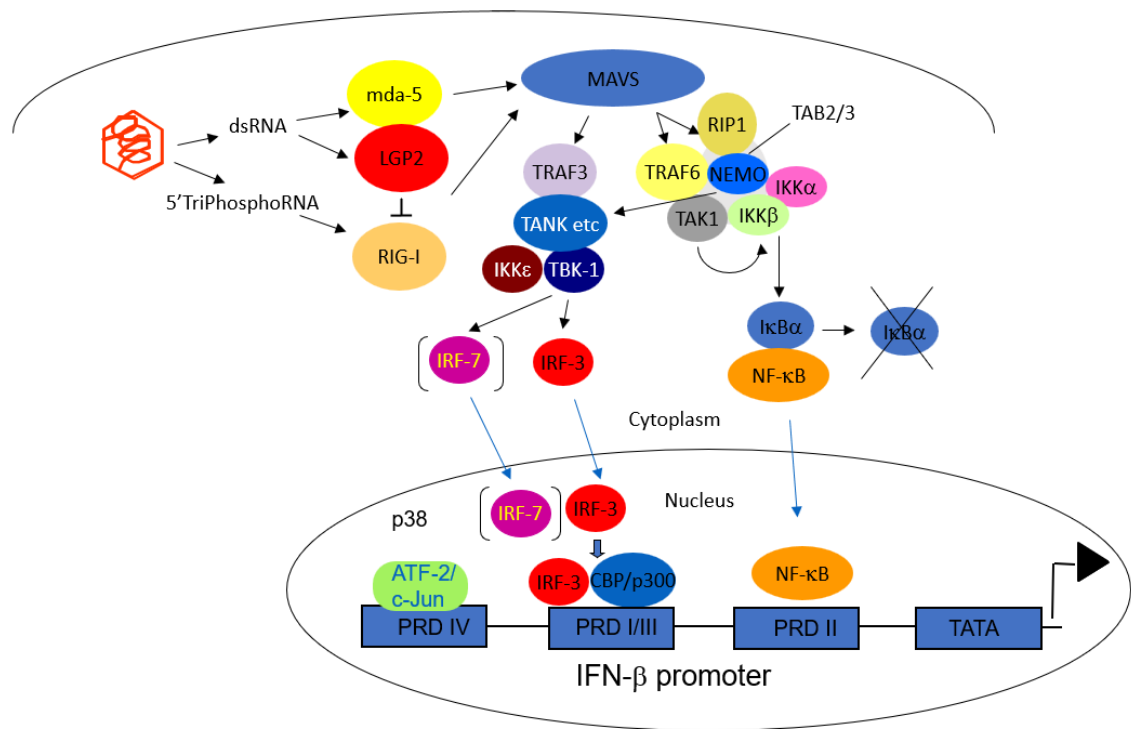


Figure 1.6 - The RLR-dependent induction of IFN. dsRNA RNA is detected by the cytosolic PRRs RIG-I and MDA5 which, upon activation, recruit the MAVS adaptor protein. MAVS then recruits multiple signalling components such as TRAF3 and TBK-1, which in turn phosphorylates IRF3 (and IRF7 when present). Phosphorylated IRF3 (or IRF7) is translocated to the nucleus where it binds to the IFN- β promoter and induces the expression of IFN- β . Figure adapted from Randall & Goodbourn 2008⁸⁶.

The genome of -ssRNA viruses bears a 5'ppp structure, and during replication the polymerase occasionally switches template onto the nascent RNA so that a 'copy-back' defective viral genome (DVG) is produced¹¹⁶. DVGs have long self-complementary sequences which form a strong hairpin structure with a blunt end, which is a ligand for RIG-I and when detected results in the induction of IFN¹¹⁷. Copy-back DVGs are shorter than full-length non-defective genomes and are replicated from the strong Tr promoter, giving them a replicative advantage which can interfere with replication of viable virus genomes^{118,119}. Consequently, the production of DVGs by the viral polymerase is likely to be highly regulated, as evidenced by the lack of IFN induction in most cells infected by paramyxoviruses¹²⁰. It is not clear whether the production of DVGs is simply an unavoidable process in viral RNA synthesis, or whether their production has evolved to regulate viral replication in a manner beneficial to the virus, for example by promoting persistent infection^{121,122}.

1.2.2 Interferon-stimulated genes (ISGs)

Type I and type III IFN genes are induced downstream of the activation of PRRs and the encoded IFN cytokines are secreted from the infected cell. Type I IFNs are the most potent and their receptor, the IFN- α/β receptor (IFNAR), is expressed on the surface of most cells. Binding of IFN- α/β to IFNAR activates the IFNAR-associated tyrosine kinases JAK1 and TYK2, which phosphorylate both STAT1 and STAT2, leading to the formation of STAT1/STAT2 heterodimers. The STAT1/STAT2 heterodimer is translocated to the nucleus and interacts with IRF9 to form the IFN-stimulated gene factor 3 (ISGF3). ISGF3 binds to IFN-stimulated response elements (ISREs) in the promoters of hundreds of IFN-stimulated genes (ISGs) which have numerous antiviral functions^{123–126}. Examples of ISGs include PKR, 2'-5'-OAS, IFIT1, and MX1. A diagram of IFN signalling is shown in figure 1.7. A subset of ISGs, including IFIT1, are also induced directly by RLR/MAVS/IRF3 signalling in an IFN-independent manner^{86,127}.

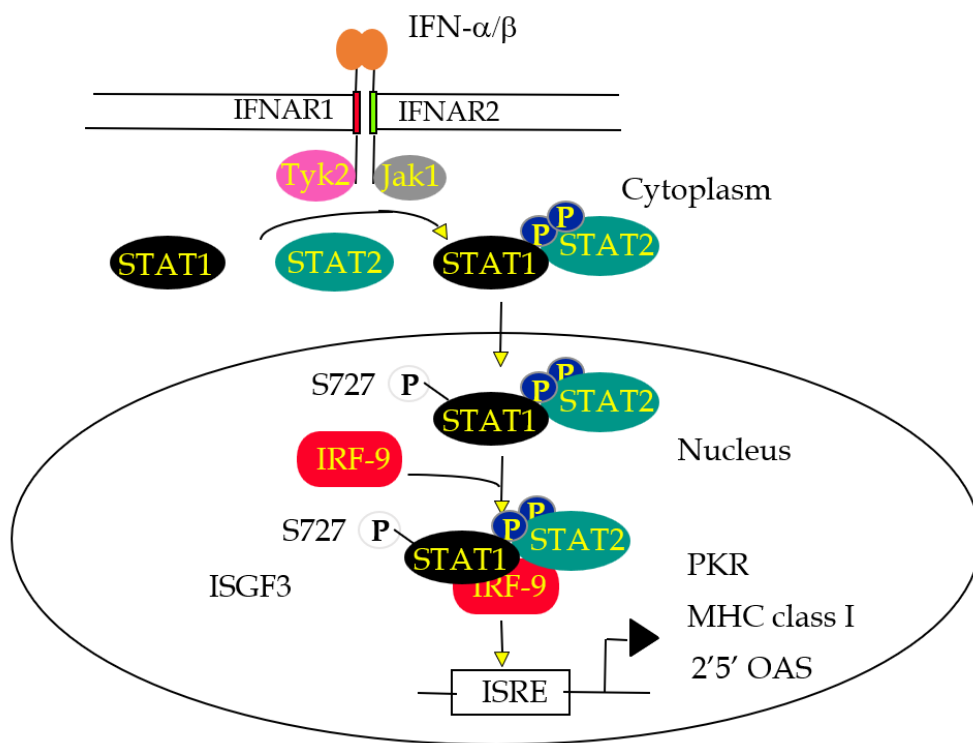


Figure 1.7 – Type I IFN signalling and the induction of ISGs. IFN- α/β is secreted from the infected cell and binds IFNAR. Activation of the IFNAR-associated kinases JAK1 and TYK2 leads to phosphorylation of STAT1 and STAT2, inducing their heterodimerisation. The STAT1/STAT2 heterodimer is translocated to the nucleus and interacts with IRF9 to form the IFN-stimulated gene factor 3 (ISGF3). ISGF3 binds to IFN-stimulated response elements (ISREs) in the promoters of ISGs and induces their expression. The gene products of the many hundreds of ISGs include PKR, 2'-5'-OAS, IFIT1, and MX1. Figure adapted from Randall & Goodbourn 2008⁸⁶.

Some ISGs have a global effect on cellular processes. These include PKR, which is activated by dsRNA and phosphorylates eIF2 α , inhibiting all translation¹²⁸; and 2'-5'-OAS, which is also activated by dsRNA and produces ligands which activate RNase L, leading to non-specific degradation of RNA¹²⁹. ISGs which have a specific effect on RNA virus replication include the following: IFIT1, which inhibits the translation of viral mRNAs by binding to the 5' cap¹³⁰; ZAP, which recognises viral RNA (potentially due to elevated CpG content) and targets it for degradation¹³¹; ISG20, which decreases translation of exogenous 'non-self' transcripts by an unknown mechanism^{132,133}, possibly as a result of recognition of internal m6A modifications¹³⁴; PARP12, which interacts with the cellular translation machinery to reduce translation of viral transcripts^{135,136}; Viperin, which generates 3'-deoxy-3',4'-didehydro-CTP which acts as a chain terminator when incorporated by viral RNA polymerases¹³⁷; and MX1, which binds to and disrupts viral nucleocapsid proteins¹³⁸. ISGs can also interfere with virus cell entry (e.g. IFITM3¹³⁹) and the release of virus particles from cells (e.g. tetherin¹⁴⁰).

1.2.3 Viral evasion of the IFN system

The ability of ISGs to restrict viral replication has led to the evolution of numerous viral strategies to both avoid induction of IFN and to disrupt IFN signalling once it has been produced. A range of such innate immune evasion strategies employed by multiple virus families are shown in figure 1.8.

For paramyxoviruses, evasion of the IFN system is primarily achieved by either avoidance of the generation of DVGs or the disruption of IFN induction and signalling by viral accessory proteins. These accessory proteins are produced by mRNA editing of the P/V ORF, as previously described and shown in figure 1.2C. The V accessory protein of multiple paramyxoviruses can interact via its C-terminus with MDA5 to prevent activation by dsRNA^{6,141–143}. V can also potentially limit the activation of RIG-I via its interaction with LGP2¹⁴⁴. The V protein of PIV5 disrupts IFN signalling by targeting STAT1 for degradation⁸; this process is dependent upon the binding of V to STAT2 and the subsequent recruitment of DDB1 and Cul4a to the STAT1/STAT2 heterodimer, leading to the ubiquitination and degradation of STAT1⁷. A similar mechanism of STAT degradation is employed by other paramyxoviruses¹⁴⁵. Other paramyxovirus accessory proteins produced by mRNA editing include the W proteins of henipaviruses, which also serve as innate immune antagonists¹⁴⁶. Finally, some paramyxovirus genera produce a C protein from alternative start codons either upstream or downstream of the P start codon (i.e. not by mRNA editing). C

proteins have been shown to limit the induction of IFN by either inhibiting the activation of RIG-I¹⁴⁷ or by regulating viral RNA synthesis to reduce PAMP production¹⁴⁸.

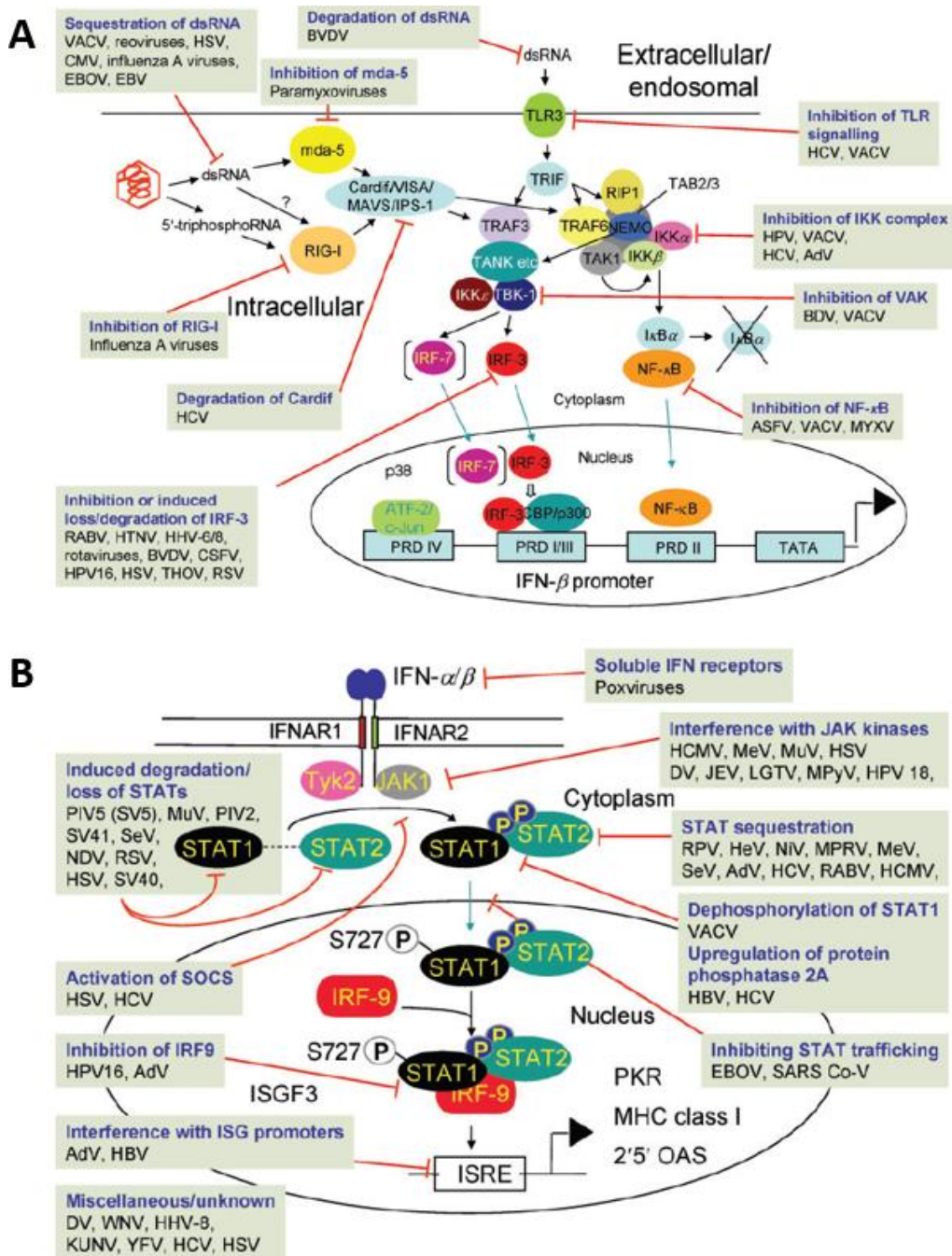


Figure 1.8 – Disruption of IFN induction (A) and IFN signalling (B) by the innate immune antagonist proteins encoded by various viruses. For each listed virus, the component of the signalling pathways and the mechanism of interference is shown. Figure adapted from Goodbourn & Randall 2008⁸⁶.

1.3 IFIT proteins and mRNA cap structure

Previous work by the Goodbourn and Randall groups identified Interferon-induced protein with tetratricopeptide repeats 1 (IFIT1, also known as ISG56 and P56) as the ISG primarily responsible for the sensitivity of PIV5 to human IFN^{149,150}. One of the main aims of this project will be to investigate the mechanisms of this inhibition and to explore the consequences for of this on viral fitness. This section briefly summarises the current literature on IFIT1.

1.3.1 The Interferon-induced protein with tetratricopeptide repeats (IFIT) family

IFIT1 is a 56 kDa protein for which the cDNA was first cloned in 1983 as part of an effort to identify IFN-inducible genes¹⁵¹. Subsequent studies have found it to be among the most-expressed ISGs in human cell lines with copies per cell reaching $1.4\text{--}2.4 \times 10^6$ following treatment with IFN- β ¹⁵². The protein can be detected within 4h of induction by IFN, with maximal levels being reached 8-24h post-induction¹²⁷. IFIT1 is highly expressed in response to type I IFNs, but only minimally in response to type II interferon^{153,154}, and is induced by a wide range of viruses during infection^{155–157}.

The human IFIT1 gene is located on chromosome 10 as part of the human IFIT gene locus which also encodes IFIT2 (ISG54), IFIT3 (ISG60), IFIT5 (ISG58), and the non-IFN-inducible IFIT1B^{158,159}. IFIT genes are found in most vertebrates, including mammals and fish, and have expanded by lineage- and species-specific gene duplication, having most likely originated from a vertebrate ancestor around 450 million years ago¹⁶⁰. Gene deletions and recombination have resulted in large variation in the number of different IFIT genes between species. For instance, several species have lost the IFIT1B gene (including chimpanzees, marmosets, cows, sheep, pigs, and bats), others have lost the IFIT1 gene (primarily rodents, including mice and rats), while birds have a reduced number of IFIT genes either due to gene deletions or reduced gene duplication^{160,161}. In some species such as cats, dogs, and rabbits, recombination has occurred between IFIT1 and IFIT1B¹⁶¹.

As the name suggests, the IFIT family proteins contain a series of tetratricopeptide repeat (TPR) structural motifs. TPRs consist of repeated sequences of 34 amino acids that mediate protein-protein interactions. As may be expected for proteins bearing TPRs, human IFIT1, IFIT2, and IFIT3 have been found to interact with each other and many host proteins involved in RNA-binding¹⁵². These interactions are also mediated by the dimerization of IFIT1 via a conserved region of

the C-terminus¹⁶². This network of interactions has been termed the 'IFIT interactome' and serves to regulate the function of these proteins¹⁶³.

1.3.2 Antiviral functions of IFIT1

Reported antiviral functions of IFIT1 include non-specific inhibition of translation by binding to translation initiation factor eIF3^{164–168}; sequestration of viral proteins¹⁶⁹; and positive regulation of the IFN response^{170–174} (though in contrast reports also exist of a negative-feedback role for IFITs^{175,176}). These functions likely involve protein-protein interactions mediated by the TPR motifs of IFIT proteins.

In addition to these mechanisms, research over the last decade has revealed IFIT1 to be an RNA-binding protein capable of distinguishing between host 'self' RNA and viral 'non-self' RNA. Firstly, IFIT1 was found to bind uncapped 5' triphosphorylated RNA (ppp-RNA)^{152,177}, a structure found on the genomes of -ssRNA viruses. IFIT1 binding to viral genomes in this manner can presumably inhibit viral replication either by displacing viral proteins from the genome or by sequestering the genome.

However, more recent work has identified the primary target of IFIT1 as cap0 mRNA^{130,178–180}, which differs from cap1 mRNA in the methylation status of the first transcribed nucleotide. All eukaryotes generate mRNA which is capped with an inverted N7-methylated guanosine (m7G), linked by a 5'-5' triphosphate bridge^{181,182}. This is referred to as 'cap0' and the m7G cap is necessary for mRNA stability¹⁸³ and translation¹⁸⁴. In higher eukaryotes¹⁸⁵, mRNA is additionally methylated at the 2' hydroxyl of the ribose of the first transcribed nucleotide by cap methyltransferase 1 (CMTR1¹⁸⁶) to form a 'cap1' structure. A portion of transcripts are then further 2'-O-methylated at the ribose of the second transcribed nucleotide by CMTR2¹⁸⁷ to form a 'cap2' structure. IFIT1 is able to bind to cap0 structure and outcompete the binding of translation initiation factor eIF4E, thus inhibiting translation of the mRNA it is bound to. A diagram showing the various methylations of eukaryotic mRNA cap structure is shown in figure 1.9.

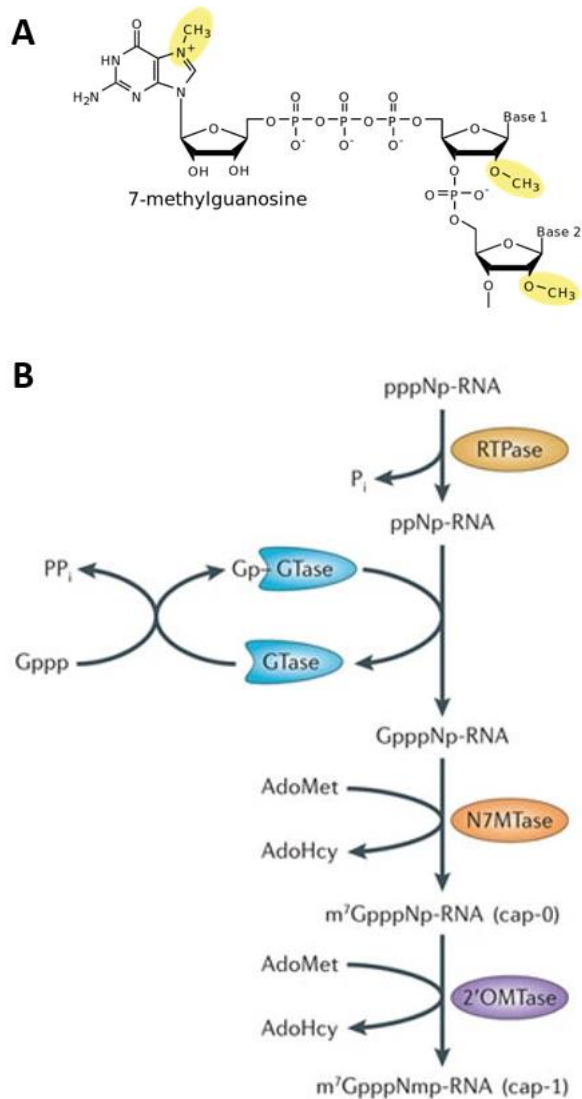


Figure 1.9 - The cap structure of eukaryotic mRNA. A) Cap structure and B) enzymatic reactions. The guanosine cap is methylated at the N7 position by a N7MTase to form 7-methylguanosine (m⁷G) and is linked via a 5'-5' triphosphate bridge to the first transcribed nucleotide, forming the minimal cap₀ structure. In higher eukaryotes, the first nucleotide is methylated by the 2'OMTase CMTR1 at the 2' hydroxyl to form cap₁ structure. CMTR2 methylates the second nucleotide to form cap₂ structure. Methyl groups are highlighted in yellow. Figure adapted from Decroly et al. 2012¹⁸⁸ and Wikipedia Commons: https://commons.wikimedia.org/wiki/File:Cap_Structure.svg.

In contrast to the m⁷G cap₀ structure, cap₁ and cap₂ methylations are not required for translation of mRNA in most conditions^{186,189}. While CMTR1 is localised to the nucleus and acts on all cellular transcripts, CMTR2 is cytoplasmic and non-specifically methylates transcripts so that the cap₂-methylation status of mRNAs is a function of their age¹⁹⁰, resulting in around 50% of cellular transcripts bearing cap₁ methylation alone and 50% bearing both cap₁ and cap₂ methylations. Despite being proposed to have roles in, for example, the regulation of mRNA localisation¹⁹¹, the main evolved function of cap₁ and cap₂ structures may be to serve as a 'self' structure that differentiates host mRNA from viral mRNA, enabling the specific restriction of viral translation by IFIT1 or the induction of IFN in response to viral infection^{190,192}. The current

model of how IFIT1 inhibits the translation of incompletely methylated viral mRNAs is shown in figure 1.10.

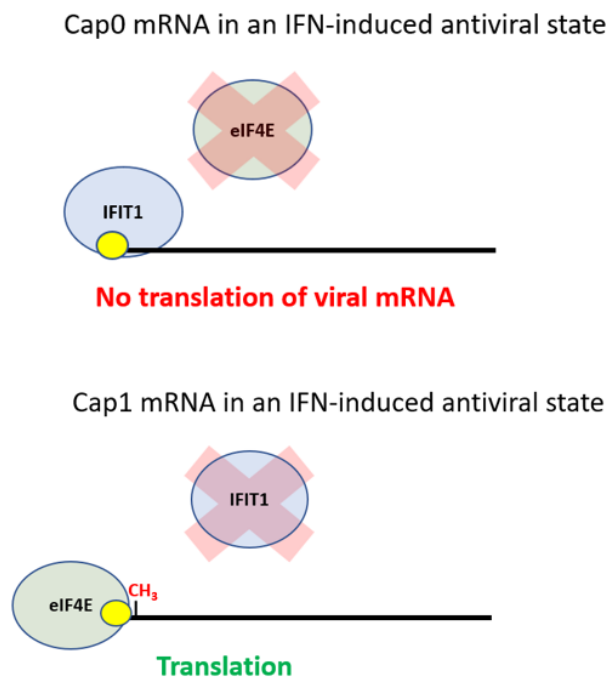


Figure 1.10 - Inhibition of viral translation by IFIT1. Capped (yellow) viral mRNA that lacks methylation at the first nucleotide (cap0) is bound by IFIT1 with high affinity, thus excluding the cap-binding translation factor eIF4E and preventing translation. In contrast, cellular mRNA is methylated at the first nucleotide (cap1), preventing the binding of IFIT1 but not eIF4E, thus allowing translation.

1.3.3 Viral strategies to evade IFIT1

The ability of IFIT1 to distinguish self from non-self RNA and to selectively inhibit incompletely methylated transcripts produces a strong selection pressure on viruses. Any virus that fails to generate a 'self' mRNA cap1 structure risks restriction by IFIT1, and unsurprisingly, most human viruses have evolved mechanisms to evade its antiviral function so that they are not inhibited by it¹⁹³. The variety of strategies employed by viruses to evade IFIT1 are summarised in this section.

For viruses that replicate in the nucleus, the simplest method of IFIT1 evasion is to utilise host enzymes for transcription and mRNA capping. Such viruses include members of the *Herpesviridae*, *Adenoviridae*, *Papillomaviridae*, *Polyomaviridae*, *Parvoviridae*, *Bornaviridae* families (all DNA viruses), and also *Retroviridae* (RT-RNA viruses). Host polymerases and methyltransferases will produce mRNA with cap1 or cap2 structure, neither of which is bound by IFIT1.

Viruses that replicate in the cytoplasm and rely on cap-dependent translation must encode their own transcription and capping enzymes, and most cytoplasmic viruses have evolved to encode their own methyltransferases (MTases) to generate cap1 mRNA^{188,194,195}. These include the *Paramyxoviridae*, *Rhabdoviridae*, *Filoviridae*, *Flaviviridae* (excluding the *Hepacivirus* genus), *Coronaviridae*, and *Reoviridae* (all RNA virus families), and also the DNA virus family *Poxviridae*. Viral cap1 MTases share some homology with cellular MTases¹⁹⁴, and the cap structures they generate enable evasion of IFIT1. Despite sharing homology with cellular MTases, the sequential order of methylations by *Mononegavirales* L proteins has been suggested to be the reverse of that in eukaryotes (shown in figure 1.9B); for example, Vesicular stomatitis virus (VSV, family *Rhabdoviridae*) cap1 methylation precedes and is required for subsequent m7G methylation^{196–198}. In contrast, positive sense RNA viruses such as the *Flaviviridae* and *Coronaviridae* methylate in the more canonical order shared by eukaryote MTases^{178,199}.

Several cytoplasmic RNA virus families (e.g. *Picornaviridae*, *Caliciviridae*, and the *Hepacivirus* genus of *Flaviviridae*) encode an internal ribosome entry site (IRES) in the 5'UTR of the +ssRNA genome. IRESs recruit translation factors to enable cap-independent translation^{200,201}. The 5' end of these +ssRNA genomes is masked by a covalently bound viral protein²⁰², but binding of IFIT1 to the 5' end of these genomes would have no effect on IRES-dependent translation anyway. However, IFIT1 has been found to inhibit translation from the Hepatitis C virus (HCV) IRES, possibly by preventing the recruitment of eIF3²⁰³.

Some RNA virus families – *Orthomyxoviridae*²⁰⁴, *Hantaviridae*, and *Arenaviridae*²⁰⁵ – employ the mechanism of 'cap-snatching' to generate their mRNA cap structures. The viral polymerase directs the cleavage of host mRNA 10-20 nucleotides downstream of the 5' cap; this short sequence is used to initiate transcription of viral mRNAs so that they contain an authentic 'self' mRNA cap1 or cap2 structure. Cap-snatching occurs either in the nucleus (*Orthomyxoviridae*) or cytoplasm (*Hantaviridae* and *Arenaviridae*), and results in transcripts which cannot be bound by IFIT1.

Finally, viruses of the *Alphavirus* genus (cytoplasmic +ssRNA viruses of the *Togaviridae* family) neither encode a cap1 MTase nor have an IRES, and do not engage in cap-snatching. The cap0 mRNA structure generated by these viruses²⁰⁶ means they are likely to be sensitive to IFIT1; indeed the replication of Sindbis virus (SINV), Venezuelan Equine Encephalitis virus (VEEV), and Chikungunya virus (CHIKV) have been found to be inhibited by IFIT1^{123,207}. However, while a subsequent study found SINV strain Girdwood to be sensitive to IFIT1, O'nyong'nyong virus (ONNV) and SINV strain AR86 were found not to be¹²⁵. The varying sensitivity of alphaviruses to IFIT1 has recently been mapped to differences in the RNA secondary structure of the viral

5'UTR^{208,209}. Hairpin structures that result in the pairing of cap-proximal bases were found to limit the binding of IFIT1, which requires a single-stranded 5' overhang of at least 4 nt^{179,180}.

1.4 Aims

This project will focus on two aspects of PIV5 biology: the impact of mutations in the P protein subunit of the viral polymerase on RNA synthesis, and the sensitivity of the virus to the IFN-stimulated gene IFIT1. The mutations in P form a short section at the beginning of chapter 3, while the sensitivity of the virus to IFIT1 became the main focus of the project and forms the basis of the remainder of chapter 3 and chapter 4. Chapter 5 investigates the properties of IFIT proteins from different species which may be relevant to the host tropism of paramyxoviruses.

During the course of this project, it became apparent that both themes (P mutations and IFIT1 sensitivity) are potentially connected to the ability of PIV5 to form persistent infections, as discussed in section 1.1.4. Therefore, the discussion (chapter 6) focusses on how the findings presented in chapters 3-5 may reveal potential mechanisms of PIV5 persistence.

The specific aims of this project are as follows:

- To investigate the impact of changes in the PIV5 P protein on viral polymerase activity. Some of these have been previously characterised, but this analysis will be expanded to include a wider range of changes which vary between strains of PIV5. Different strains are known to have different replication dynamics, so the aim is to link phenotypic differences to genotypic changes in the P gene. This work forms the first half of chapter 3.
- To further characterise the sensitivity of PIV5 to IFIT proteins. With previous work from the Randall and Goodbourn groups implicating IFIT1 as the main ISG responsible for the sensitivity of PIV5 to IFN, a more thorough investigation of the both the causes and consequences of this sensitivity is warranted. In particular, the sensitivity of different paramyxoviruses to a variety of IFIT proteins will be tested along with the role of differences between the viral methyltransferases (L proteins) in determining whether each virus is sensitive to IFITs. This work forms the remainder of chapter 3.
- To understand the basis for the differing sensitivity of PIV5 mRNAs to IFIT1. Multiple publications from the Randall and Goodbourn groups have shown that the extent to

which the level of each PIV5 protein is affected by IFN/IFIT1 varies, but the reason for this is unknown (see the beginning of chapter 4 for a more detailed explanation). The potential for different viral transcripts to have differing affinities for IFIT1 will be investigated using a variety of techniques. This work forms chapter 4.

- To compare the cap-binding properties of IFIT proteins from different species. The impact of various mRNA cap-proximal methylations on IFIT binding will be assessed using a yeast growth assay. This work forms chapter 5 and is also informed by findings presented in chapter 3. sented in chapter 3.

Chapter 2 – Methods

2.1 Cell culture

All cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 4.5 g/L Glucose, with L-Glutamine (Lonza BE12-604F) with 10% v/v Foetal Bovine Serum (FBS) (Sigma F7524) and 1% v/v Penicillin/Streptomycin (Sigma P4458), except BSRT7 for which 5% FBS was used, and DF-1 cells for which chicken serum (Sigma) was additionally added to 2%. Infections were carried out in the same as above but with 2% FBS. Cells were incubated at 37°C and 10% CO₂ except for DF-1 cells, which were incubated at 39°C and 5% CO₂. For passaging cells as required, the monolayer was washed with PBS (Sigma, 806552) and trypsinised (Trypsin/EDTA (Sigma, T3924)) before being resuspended in fresh medium. To make frozen stocks, pelleted cells were resuspended in 1ml freezing medium (FBS +10% DMSO v/v), transferred to cryovial, and slowly frozen at -80°C prior to being transferred to liquid nitrogen storage. Frozen stocks were thawed rapidly at 37°C, diluted with standard medium, pelleted, and then resuspended in standard medium and transferred to the required tissue-culture plate.

Cell lines used: 293 (human embryonic kidney cells, ATCC CRL-1573); 293 IRF3ko (made using CRISPR-Cas9 by Maite Pérez Cidoncha, SGUL); 293 IFIT1ko (made during this project using CRISPR-Cas9); Vero (African green monkey kidney cells, ATCC CCL-81); Vero-V (Vero cells stably expressed PIV5-V, made by Emma Poole, SGUL); BSRT7 (BHK-21 cells stably expressing T7 RNA polymerase²¹⁰); A549 (human lung epithelial cells, ATCC CCL-185); A549/pr(IFN-β).GFP (A549 cells with GFP under control of the human IFN-β promoter¹²⁰); A549.GFP IFIT1ko (made during this project using CRISPR-Cas9); BALB/3T3 (mouse fibroblast cells, ATCC CCL-163); BALB/3T3 mIFIT1ko (made during this project using CRISPR-Cas9); DF-1 (chicken embryonic fibroblast cells, ATCC CRL-12203).

All plastic tissue-culture-treated dishes and plates (10cm plates, 6cm plates, 6-well plates, 12-well plates, 24-well plates, 96-well plates, T75 flasks) were supplied by Corning.

2.2 Transfections

Transfection of 293 cells (at 50-80% confluence) was performed using acidified polyethylenimine (PEI) pH 4²¹¹. DNA was diluted to 20ng/μl in lactate-buffered saline (LBS) and PEI diluted 1:50, also in LBS. Having combined the diluted plasmid DNA in the desired ratio, an equal amount of diluted PEI was added and the mixture was left at room temperature for 15 minutes. The

transfection mixture was then added to cells without changing the medium. At 6h post-transfection, the medium was replaced.

For virus rescue transfections in BSRT7 cells, Lipofectamine LTX with Plus Reagent (ThermoFisher, A12621) was used. A total of 1700ng of DNA was added to 150µl serum-free DMEM followed by 1.7µl Plus reagent and 10µl Lipofectamine LTX, and then left at room temperature for 5 minutes. Cell culture medium was refreshed prior to addition of the transfection mixes.

2.3 CRISPR-Cas9-mediated gene knockout

CRISPR-Cas9 sgRNAs were expressed from pSpCas9(BB)-2A-GFP and pSpCas9n(BB)-2A-Puro. sgRNAs were designed to be offset by a short sequence (7bp for hIFIT1 and 1bp for mIFIT1) to reduce off-target activity along with a Cas9 nickase mutant²¹². The binding sites of the sgRNAs used for knockout of hIFIT1 and mIFIT1 are shown below. The second site of each sgRNA is given as the top strand sequence despite the sgRNA being the reverse complement.

hIFIT1: GAATGAGGAAGCCCTGAAGA and AGAAGCTGAAAACCTTAATGC

mIFIT1: GTTCCTTGACATCAAGAACC and ATTGGGGATGCACAACCTCC

Cells were transfected in 6-well plates with 500ng of each plasmid per well. At 24 hours post-transfection, transfection was confirmed by checking GFP expression and the cells were then trypsinised, resuspended and serially diluted into 10cm tissue-culture plates. 24 hours later, puromycin was added (2µg/ml for 293 cells, 4µg/ml for A549 cells, and 7µg/ml for BALB/3T3 cells). Plates were left until colonies became visible at which point cloning rings (Sigma, C7983) with bases covered in silicone grease (Dow-Corning, Z273554) were placed around them. Trypsin was added and the detached cells transferred to a well of a 24-well plate. Cells were grown out and harvested for both Western blot (having been treated with IFN) and sequence analysis. Genomic DNA was isolated using DNAzol reagent (Invitrogen, 10503027) and the DNA dissolved in 8mM NaOH. A short sequence (150-200 bp) including the edit site was amplified by PCR and the products analysed on a 2% agarose gel to check for insertions or deletions. PCR products were gel-purified and sequenced.

2.4 Viruses

Viruses were recovered from pBH276, a full-length cDNA PIV5 clone which can be used to generate the viral genome from a T7 promoter²¹³. Per well of a 6-well plate seeded with BSRT7 at 50-70% confluence, 1000ng pBH276, 100ng pCAGGS-NP, 100ng pCAGGS-P(CPI+), and 500ng pCAGGS-L were transfected using Lipofectamine LTX as detailed in the transfection section. At

2 days post-transfection, medium was replaced with DMEM/2% FBS. Supernatant was harvested at 5-6 days post-transfection a small amount (e.g. 20µl) used to infect near-confluent Vero cells in a T75 flask in DMEM/2%. At 5 dpi, supernatant was aliquoted to microcentrifuge tubes, flash-frozen in liquid nitrogen, and stored at -80°C.

Modifications were made to pBH276 using HiFi Assembly. Briefly, PCR fragments between the AgeI site in NP and the SphI site in F were generated containing the desired mutations. PCR products were then assembled with an AgeI/SphI backbone generated by restriction digest. The sequence of the mutation and the PCR-generated section of the cloned plasmids was confirmed by sequences.

To titre virus stocks of mCherry-expressing viruses, plaque assays were performed. Vero cells were seeded for near-confluence in 24-well plates. Virus stock was serially diluted 1/10 in serum-free DMEM. Cell culture medium was aspirated and replaced with 500µl serum-free DMEM. 100µl of each virus dilution (typically those between 10^{-2} and 10^{-8}) was added to a well and incubated for 1 hour. Cell culture medium was then aspirated and 500µl of overlay (DMEM/2%FBS, 0.4% Avicel) added. At 4 dpi, overlay was aspirated, the cells were washed once with PBS. mCherry plaques (colonies) were visualised under a fluorescent microscope and counted.

2.5 Sequence information

The GenBank accession numbers of PIV5 strains referenced in this project are shown below:

PIV5 strain	GenBank accession no.
W3A	JQ743318
AGS	KX060176
CPI+	JQ743321
CPI-	JQ743320
DEN	JQ743322
H221	JQ743323
D277	KC237065
1168-1	KC237064
SER	JQ743328
KNU-11	KC852177

The GenBank accession number for each of the L proteins aligned in figure 3.22 are shown below:

Virus	L protein GenBank no.
CDV	NP_047207.1
RPV	YP_087126.2

MEV	NP_056924.1
HEV	NP_047113.3
NIV	NP_112028.1
SEV	NP_056879.1
HPIV3	NP_067153.2
PPIV1	YP_009094450.1
HPIV2	NP_598406.1
SV41	YP_138510.1
PIV5	YP_138518.1
MUV	NP_054714.1
HPIV4A	YP_008378665.1
MPRPV	YP_001249278.1
PORPV	YP_001331035.1
NDV	NP_071471.1
APMV6	NP_150063.1
VSV	NP_041716.1
RABV	NP_056797.1
EBOV	NP_066251.1
MARV	YP_001531159.1

The GenBank accession number for each of the L proteins aligned in figure 3.27 are shown below:

Virus	L protein GenBank no.
NDV	NP_071471.1
APMV2	YP_009177238.1
APMV3	YP_009094217.1
APMV4	YP_007008152.1
APMV5	YP_009094160.1
APMV6	NP_150063.1
APMV7	YP_009094060.1
APMV8	YP_009094225.1
APMV9	YP_009094364.1
APMV10	YP_009094073.2
APMV11	YP_009094483.1
APMV12	YP_009094173.1
APMV13	YP_009255226.1
APMV15	YP_009380509.1

The GenBank accession number for the genome of each virus aligned in figure 4.1 are shown below:

Virus	Genome GenBank no.
PIV5	AF052755
PIV2	X57559

MUV	AB040874
SV41	X64275
HEV	AF017149
NIV	AF212302
MV	AB016162
CDV	AF014953
SEV	AB195968
PIV3	EU326526
NDV	AY562991
APMV6	EF569970

The GenBank accession number for each of the IFIT proteins referenced in this project are shown below:

IFIT	IFIT protein GenBank no.
hIFIT1	NP_001539.3
hIFIT2	NP_001538.4
hIFIT3	NP_001540.2
hIFIT5	NP_036552.1
hIFIT1B	NP_001010987.1
mIFIT1	NP_032357.2
mIFIT1B	NP_001103987.1
chIFIT5	NP_001307351.1
zfIFIT5	XP_002188588.5
zfIFITx	XP_004175020.3

2.6 Molecular cloning

Plasmids were generated using standard molecular biology techniques. Restriction enzymes (all New England Biolabs), T4 DNA ligase (ThermoFisher), rSAP (New England Biolabs), Q5 High-Fidelity DNA Polymerase (New England Biolabs), HiFi DNA Assembly Master Mix (New England Biolabs), and RevertAid Reverse Transcription kit (ThermoFisher) were all used according to manufacturer instructions.

For gel-purification of PCR products and restriction fragments from agarose gels (1-2% agarose, 1:10,000 ethidium bromide), the band was visualised by UV light and excised into a microcentrifuge tube. 200µl 6M NaI was added and the tube incubated at 50°C for 10 minutes. 10µl silica suspension (silica (Sigma, S-5631) in PBS) was added and mixed. Silica was pelleted by centrifugation at 16,000 xg for 1 minute and resuspended in 1ml wash buffer (50mM NaCl, 10mM Tris pH 7.5, 2.5mM EDTA, 50% v/v ethanol). Silica was re-pelleted and supernatant

removed. The silica pellet was resuspended in 10-50µl water and pelleted once again. The supernatant containing DNA was transferred to a fresh tube.

Plasmids were transformed and grown using competent DH5α *E. coli* prepared in TB buffer (10mM PIPES, 15mM CaCl₂, 250mM KCl, 55mM MgCl₂, corrected to pH 6.7 with KOH). DNA to be transformed was added to 100ul resuspended DH5α and heat shocked at 42°C for 90 seconds before being placed on ice for 2 minutes. 1ml of LB was added and the cells incubated at 37°C for 1 hour before being plated on selective 100µg/ml ampicillin (Sigma) LB agar plates. Colonies were picked and grown in LB with 100µg/ml ampicillin.

For small scale minipreps, 1ml of overnight culture was pelleted and plasmid isolated using the alkaline lysis method. For larger midipreps, 50ml of overnight culture was pelleted and plasmid isolated using the PureYield Plasmid Midiprep System (Promega). Purified plasmids were eluted and stored in TE buffer (10mM Tris, 1mM EDTA, pH 8.0) at -20°C. Plasmid concentration was determined using a NanoDrop spectrophotometer.

2.7 SDS-PAGE and Western blotting

Cells were collected in PBS by scraping and then pelleted by centrifugation at 16,000xg for 12 seconds. Pelleted cells were resuspended in lysis buffer (150mM NaCl ; 50mM Tris-HCl pH7.4; 1mM EDTA ; 1% Triton X-100) with added protease inhibitors (1mM Benzamidine; 30µg/ml Leupeptin; 5µg/ml Aprotinin; 5µg/ml Pepstatin A). Samples were rotated on a wheel for 10 minutes at room temperature and then centrifuged for 10 minutes at 16,000xg at 4°C. The supernatant was transferred to a fresh tube and the appropriate amount of 4x SDS gel loading buffer (200mM Tris-HCl pH6.8; 8% SDS; 20% 2-mercaptoethanol; 40% glycerol; bromophenol blue) was added to achieve a final concentration of 1x. Samples were heated at 95°C for 2 minutes and then stored at -20°C until used.

Prior to addition of SDS gel loading buffer, a small volume (1-3µl) of each sample was used to approximate protein concentration by Bradford assay. Samples were added to 0.15M NaCl in a final volume of 20µl in a well of a 96-well plate. 200µl of Bradford reagent (Bio-Rad) (diluted 1:5 in water) was added to each well and the absorbance at 595nm was read. In parallel, a standard curve was constructed by diluting BSA over a range of concentrations (0-8µg per well).

Gels for SDS-PAGE consisted of a 10% resolving gel (5ml 30% acrylamide (Protogel, National Diagnostics); 5.625ml 1M Tris HCl pH8.8; 4.25ml water; 75µl 20% SDS; 150µl 10% ammonium persulphate; 15µl TEMED) and a 5% stacking gel (1.67ml 30% acrylamide (Protogel, National Diagnostics); 1.25ml 1M Tris HCl pH6.8; 7ml water; 50µl 20% SDS; 100µl 10% ammonium persulphate; 10µl TEMED). Gels were run at 110V.

Following SDS-PAGE, gels were placed in ice-cold transfer buffer (3.028g Tris base; 14.413g Glycine; 200ml methanol in a final volume of 1L of water) for 10 minutes. Low fluorescence PVDF membrane (Immobilon) was wet in methanol for 15 seconds and then placed in transfer buffer. Gel and membrane were assembled in a gel sandwich and then loaded into a cassette and the tank filled with ice-cold transfer buffer along with an ice pack. Transfer was carried out at 100V with an amp limit of 0.25 for 1 hour.

Following transfer, the membrane was blocked with TBS +3% milk (Marvel) overnight at 4°C on a roller. The membrane was rinsed twice with TBS and then primary antibodies were added in TBS +3% milk +0.1 Tween-20 and incubated for 1 hour at room temperature on a roller. Following three washes with TBS +0.1% Tween-20, the secondary antibodies were added in TBS +3% milk +0.1 Tween-20 and incubated for 1 hour at room temperature on a roller in the dark. The membrane was washed twice with TBS +0.1% Tween-20 and once with TBS before being scanned on a Licor Odyssey CLx machine.

Antibodies for PIV5 (including mouse anti-V5) were obtained from Richard Randall, St Andrews University. These were used at the following dilutions: mouse anti-NP (1:1500), mouse anti-V5 (1:2000), mouse anti-M (1:1000). Rabbit anti-hlFIT1 (ThermoFisher, PA5-27907) was used at 1:1000. Rabbit anti-tubulin (Abcam, ab176560) was used at 1:1000. Mouse anti-tubulin (Sigma, T9026) was used at 1:2500. Secondary antibodies (Licor IRDye 680RD Goat anti-Rabbit and Licor IRDye 800CW Goat anti-Mouse) were both used at 1:15,000).

2.8 Minigenome assay

For each well of a 12-well plate, the following amounts of plasmid were used: 8ng MG plasmid, 30ng pCAGGS-NP, 30ng pCAGGS-P, 150ng pCAGGS-L, 150ng pCAGGS-T7pol, 32ng pCAT-LAC. The total amount of DNA was made up to 650ng using either empty pCAGGS or pEFV5. ISGs in pEFV5 were co-transfected as indicated in the results section. Amounts of plasmid were scaled up (multiplied by 3 for 6-well plates) or down (divided by 2 for 24-well plates) as required.

Cells were lysed at 48h post-transfection in 200µl Luciferase Buffer A (25mM Tris-phosphate (pH 7.8); 8mM MgCl₂; 1mM DTT; 1mM EDTA; 1% Triton X-100) and Luciferase Buffer B (same as A, plus: 30% glycerol; 800µM rATP; 2% BSA).

For each experiment, a negative control in which pCAGGS-L was excluded was used to calculate the signal/background ratio. Results are expressed as either % activity of a Wt helper protein $((100/\text{Wt SBratio}-1) * (\text{mutant SBratio}-1))$ or as % inhibition compared to pEF control $((100/\text{pEF SBratio}-1) * (\text{pEF-ISG SBratio}-1))$.

2.9 Luciferase assay

300µl of lysed sample (in Luciferase buffers A and B) was transferred to a plastic luminometer tube and luminescence read using a Lumat LB 9510 Tube Luminometer (Berthold Technologies) following injection of 100ul luciferin (6mM in water). For β -galactosidase assay, 1ml of 0.8mg/ml ONPG in LacZ buffer (60mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$; 40mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$; 10mM KCl; 1mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) was added to the same tube and incubated at 37°C. Once the colour had turned visibly yellow, absorbance was read at 420nm.

Corrected luciferase values were calculated by (raw Fluc reading – background / β -galactosidase reading – background).

2.10 Yeast

Yeast (strain PJ69-4 α) were transformed by polyethylene glycol-lithium acetate-mediated transformation. A loopful of colonies grown on YPD agar (50g/L YPD + 20g/L agar) was resuspended in 1ml YPD (50g/L YPD) and used to inoculate a 10ml culture of YPD which was grown overnight with shaking at 30°C. Cells were pelleted for 10 minutes at 2500 xg and resuspended in a small volume of YPD. This was used to inoculate a fresh 50ml culture of YPD to an OD₆₀₀ of 0.2-0.3. This was incubated with shaking at 30°C until an OD₆₀₀ of 0.4-0.6 was reached. Cells were pelleted for 10 minutes at 2500 xg and resuspended in 25ml sterile water. Cells were pelleted again and resuspended in 1ml 100mM LiOAc and transferred to a microcentrifuge tube. Cells were pelleted for 15 seconds at 16,000 xg and resuspended in a final volume of 500µl 100mM LiOAc. 50µl of this was transferred to a fresh tube for each transformation. Cells were once again pelleted and the following were added to the pellet: 240µl 50% w/v PEG3350 (Sigma); 36µl 1M LiOAc; 25µl 2mg/ml denatured ssDNA; DNA in 50µl sterile water. The pellet was resuspended by vortexing, heat shocked at 42°C for 45 minutes, allowed to recover at 30°C for 30 minutes, and then pelleted. Cells were resuspended in 100ul sterile water and spread on selective minimal synthetic defined (SD) agar plates (20g/L glucose; 19g/L agar; 6.7g/L YNB + ammonium sulphate; appropriate amino acid mix (MP Bio))

For yeast toxicity assays, cells were transformed with 1µg each of pGADT7delGAL (Leu selection), pHON1delGAL (Trp selection), and pHON7 (Ura selection). pGADT7delGAL and pHON1delGAL have had the GAL4 activation and binding domains removed. Transformations were plated on SD -Leu -Trp -Ura selective agar plates. Plates were incubated at 30°C for 3-7 days and imaged.

For yeast-2-hybrid, the genes of interest were cloned into pGADT7 and pHON1 which express genes as N-terminal fusions with the GAL4 activation domain and GAL4 binding domain,

respectively. Cells were transformed with 1µg of each plasmid and plated on both SD -Leu -Trp and SD -Leu -Trp -His selective agar plates with concentrations of 3-aminotriazole (3-AT) at 2mM, 5mM, and 10mM. Plates were incubated for 5 days at 30°C and imaged.

2.11 RNA structure predictions

Predictions were primarily made using the RNAfold WebServer (<http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi>) with the default parameters. Co-transcriptional structure predictions of full-length mRNA were made using the CoFold algorithm (<https://e-rna.org/cofold/>) with the default parameters²¹⁴.

2.12 Protein purification

The CDS of the proteins to be expressed were cloned into pET.His6SumoTEVLIC in frame with the His6 N-terminal tag. *E. coli* strain BL21(DE3) were transformed with pET.His6-IFIT and ampicillin-resistant colonies used to inoculate a 6ml LB culture. This was grown at 37°C with shaking until an OD₆₀₀ of around 0.6 was reached. At this point, the culture was split in two and either uninduced or induced with IPTG to a final concentration of 50µM and then incubated for a further 3-5 hours. 500µl of each culture was pelleted, resuspended in 50ul water + 50ul 2X SDS gel loading buffer, heated to 95° for 2 minutes, and run as detailed in the SDS-PAGE section. The gel was stained by Coomassie protein stain (10% v/v acetic acid; 45% v/v methanol; 2.5mg/ml Coomassie Brilliant Blue R250) for 1 hour and then destained (10% v/v acetic acid; 45% v/v methanol) for 1 hour. Once production of IFIT proteins had been confirmed (by comparing induced to uninduced samples), the process was repeated with a larger initial culture (1L of LB) but pellets were stored at -80°C rather than being analysed by SDS-PAGE.

Thawed pellets from above were resuspended in 6.5ml lysis buffer (50mM sodium phosphate; 300mM NaCl; 0.05% v/v Tween-20; 10mM imidazole; adjusted to pH 8.0 with NaOH) containing a small amount of lysozyme and protease inhibitors (1mM Benzamidine; 30µg/ml Leupeptin; 5µg/ml Aprotinin; 5µg/ml Pepstatin A). Samples were left on ice for 15 minutes and then sonicated for 30 seconds with 1 second pulses, amplitude 60%. Samples were centrifuged at 50,000 xg for 20 minutes at 4°C using pre-chilled tubes and rotor. The magnetic beads were prepared by adding 200µl Pierce Ni-NTA Magnetic Agarose Beads (ThermoFisher, 78605) bead slurry to a 15ml tube, adding 800µl equilibration buffer (50mM sodium phosphate; 300mM NaCl; 0.05% v/v Tween-20; 10mM imidazole; adjusted to pH 8.0 with NaOH), vortexing for 10 seconds, and discarding the supernatant following separation of the beads using a magnetic stand. This was then repeated using 1ml of equilibration buffer. The sample supernatant was

then added to the prepared beads and mixed on an end-over-end rotator for 1 hour at 4°C. The beads were collected using a magnetic stand and washed with 1ml wash buffer (50mM sodium phosphate; 300mM NaCl; 0.05% v/v Tween-20; 15mM imidazole; pH adjusted to 8.0 with NaOH) followed by a second wash with higher imidazole concentration (50mM). Washed beads were resuspended in 1ml elution buffer (50mM sodium phosphate; 300mM NaCl; 0.05% v/v Tween-20; 300mM imidazole; pH adjusted to 8.0 with NaOH) and mixed on a rotator for 10 minutes. The beads were separated once more and the supernatant transferred to a fresh tube. 6ml of desalting elution buffer (20mM Tris pH 8.0, 150mM NaCl, 1mM DTT, 5% v/v glycerol) was added and the sample was transferred to an Amicon Ultra-15 Centrifugal Filter Unit (Merck, UFC903024) with a 30 KDa cut-off and centrifuged for at 4000 xg for 20 minutes at 4°C. 8ml desalting elution buffer was added and the filter units centrifuged again. The remaining liquid above the filter was increase in volume to about 1ml, transferred to microcentrifuge tubes in 50µl aliquots, and stored at -80°C.

2.13 In vitro transcription and RNA modifications

In vitro transcription was carried out using a HiScribe T7 High Yield RNA Synthesis Kit (New England Biolabs, E2040) with 1 U/µl RiboLock RNase Inhibitor (ThermoFisher, EO0381). DNA templates (generated by PCR and gel-purified into water) were used at as large a volume as allowed in final reaction volumes of 20-30µl. For reactions with the m7GpppA cap analog (New England Biolabs, S1405), the final concentration of ATP was 2mM and of cap analog was 8mM to give a 1:4 ratio. Reactions were left for 16-24 hours at 37°C before being either run on a denaturing gel (after addition of RNA Loading Dye (New England Biolabs, B0363) and heating at 80°C for 2 minutes) or purified by Monarch RNA Cleanup kit (New England Biolabs, T2040) using the recommended 2 volumes of ethanol for small and/or structure RNA.

Denaturing gels were made using SequaGel UreaGel (7.5M urea) System (National Diagnostics). 14% 19:1 Acrylamide:Bisacrylamide gels were used to separate capped from uncapped RNA products. The gel was pre-run for 30 minutes and then run at 420V for 3-4 hours. The gel was then stained with methylene blue staining solution (1:1 0.4M NaOAc:0.4M acetic acid; 0.2% w/v methylene blue) for 15 minutes and then destained with water until bands were clearly visible. Bands were cut out and transferred to a microcentrifuge tube containing 300µl RNA gel elution buffer (20mM Tris-HCl (1M, pH 7.5); 0.25M NaOAc; 1mM EDTA pH 8.0; 0.25% w/v SDS). Samples were freeze-thawed twice between -80°C and 37°C and then left at room temperature for 48 hours. 280µl of this supernatant was transferred to a fresh tube and extracted with an equal volume of water-saturated phenol. Following centrifugation for 2 minutes, the aqueous phase was transferred to a fresh tube and purified by Monarch RNA Cleanup kit as before.

Dephosphorylation of ribozyme-generated 2',3'-cyclic phosphate was performed using T4 polynucleotide kinase (New England Biolabs, M0201) with the associated buffer in the absence of ATP for 4 hours at 37°C. Labelling of RNA with pCp-Cy5 (Jena Bioscience, NU-1706-CY5) was performed with 0.5 units/μl T4 RNA ligase (New England Biolabs, M0204); 1mM ATP; 1X T4 reaction buffer; 15% DMSO; 2μM pCp-Cy5 at 16°C for 16 hours followed by 1 hour at 37°C in the dark. Following purification RNA was diluted to 100nM.

RNA 5' Polyphosphatase (Lucigen, RP809RH) and XRN1 (New England Biolabs, M0338) were used according to manufacturer instructions.

All enzymatic reactions were carried out in the presence of 1 U/μl RiboLock and purified by Monarch RNA Cleanup kit. A NanoDrop spectrophotometer was used to measure RNA concentration and purity.

2.14 Electrophoretic mobility shift assay (EMSA)

Binding reactions were set up in a volume of 11ul containing 40nM RNA, 0.4 U/μl RiboLock, 2.2μl 5X binding buffer (75mM HEPES pH 7.4; 50mM KCl; 1mM DTT; 25mM MgCl₂; 50% v/v glycerol), 0.01mg/ml BSA, and purified protein diluted in protein elution buffer (see protein purification section). Prior to addition of other reaction components, RNA in water was heated at 70°C for 2 minutes and allowed to cool for 15 minutes. A master mix containing RiboLock, binding buffer, and BSA was added and samples left at room temperature for 15 minutes to encourage RNA folding. Protein was then added and samples left for another 30 minutes at room temperature to allow protein/RNA binding. 10ul of each sample was loaded (without loading dye) into an 8% native gel (Accugel 40%Acrylamide:Bis-Acrylamide 29:1, National Diagnostics) made with LAB buffer (10mM LiOAc, 10mM boric acid). Running buffer (LAB) was pre-chilled to 4°C and the gel was run at 250V for 45 minutes. The gel was transferred to a plastic tray in water and imaged on a ChemiDoc MP machine (Bio-Rad) using the default settings for Cy5.

2.15 In vitro translation

In vitro translation was carried out using nuclease-treated rabbit reticulocyte lysate (RRL) (Promega, L4960) in a volume of 8μl and 4.8μl RRL. RNA (generated as detailed in the in vitro transcription section) encoding Firefly luciferase was used as input at a concentration of 10mM. Protein and RNA were mixed and left for 10 minutes at room temperature prior to addition of RRL and amino acids. Reactions were incubated at 30°C for 90 minutes and then 5μl of each reaction was used for luciferase assay as detailed in the luciferase assay section.

Chapter 3 – The PIV5 minigenome system and the sensitivity of PIV5 to IFIT proteins

In this chapter, the sensitivity of PIV5 to IFIT proteins is investigated. This is primarily achieved using the PIV5 minigenome (MG) system, which is first optimised and initially used to test mutations in the P protein. The MG system is then used to study the relative impact of various IFIT proteins on viral translation, the difference in sensitivity to IFITs between PIV5, MV, and NDV, and the impact of changes in the L protein on polymerase methyltransferase activity. Finally, the impact of IFIT1 on viral growth is shown by infection of knockout cells.

3.1 Optimisation of the PIV5 minigenome system in 293 cells

Minigenomes (MGs) are useful systems for studying viral replication and RNA synthesis without having to use the virus itself^{215,216}. This is particularly advantageous for studying the effect of multiple polymerase mutants because it removes the need to go through the lengthy reverse genetics process to generate mutant viruses. MGs are also useful to study viral RNA synthesis in a controlled system (i.e. without the presence of other viral proteins which may induce cytopathic effect) and with an easily measurable readout (the reporter gene). For *Mononegavirales*, the minimal components of a MG system are the RNA polymerase L, the polymerase subunit P, the nucleoprotein NP, and a plasmid for generating the MG RNA, typically under control of a T7 promoter.

A MG system for PIV5 was first developed by the group of Biao He with chloramphenicol acetyltransferase (CAT) as the reporter gene¹⁰. The plasmid pMG-CAT contains the CAT CDS flanked by the viral Le and NP 5'UTR upstream of the start codon, and by the viral Tr and L 3'UTR downstream of the stop codon. A T7 promoter adjacent to the Tr sequence allows for transcription of the MG RNA when the plasmid is transfected into BSRT7 cells, a BHK-21-derived cell line which stably expresses T7 RNA polymerase²¹⁰. Adjacent to the Le sequence is a Hepatitis D ribozyme sequence which self-cleaves the T7 transcript and generates an authentic 3' end to the MG RNA. The 5' end of the T7 transcript has an additional GGG sequence (required for optimal T7 promoter activity) which must be removed by chance exonuclease activity for the MG RNA to conform to the 'rule of six'. The T7 transcript serves as a pseudo-genome (negative sense) and when plasmids expressing NP, P, and L are co-transfected, the RNA is

encapsidated by NP, and the regulatory Le and Tr sequences are recognised by the polymerase (L and P), resulting in transcription of the reporter gene and replication of the MG RNA. A schematic of the MG system is shown in figure 3.1.

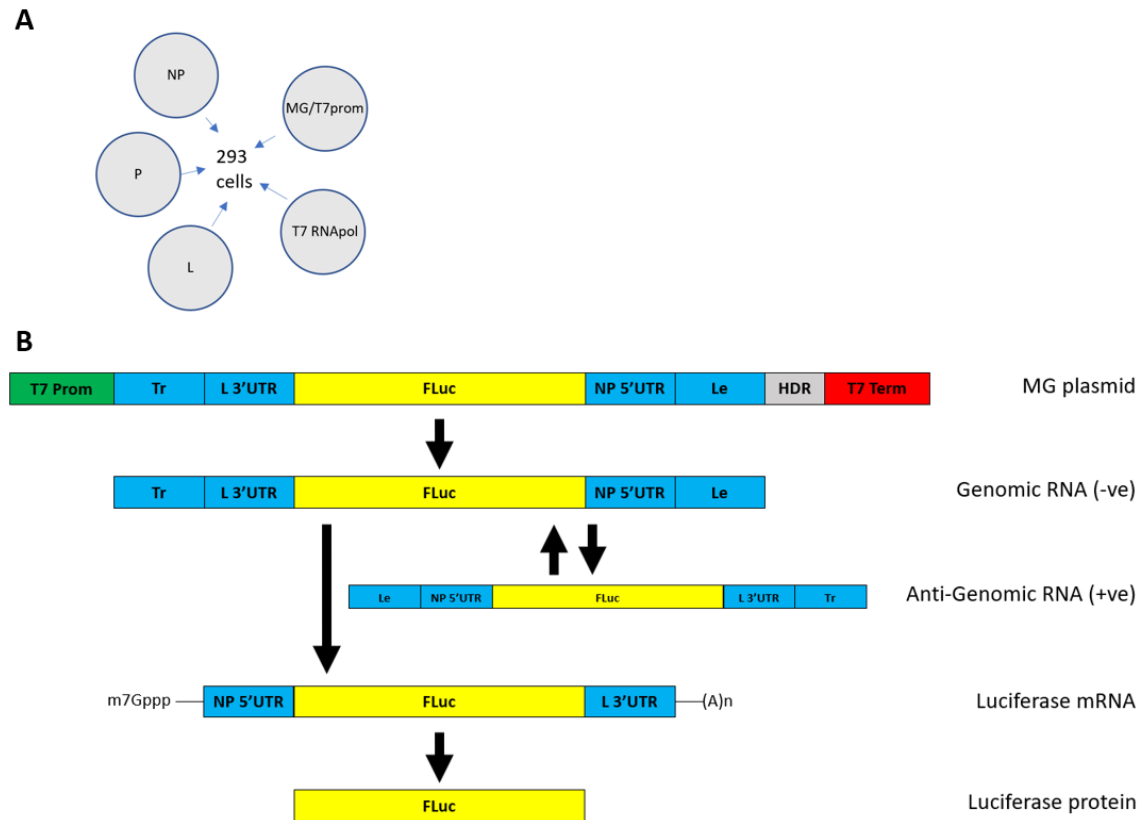


Figure 3.1 - Schematic of the PIV5 MG system with Firefly luciferase as the reporter gene. A) The minimal plasmids required for MG activity in 293 cells. B) The MG plasmid contains the sequences shown at the top: a T7 promoter (green); the Fluc CDS (yellow) surrounded by the viral Tr/L 3'UTR and Le/NP 5'UTR (blue); a Hepatitis D ribozyme sequence (grey); and a T7 terminator sequence (red). Following transcription by T7 RNA polymerase, the negative sense minigenome is encapsidated by NP and replicated (via anti-minigenome) or transcribed to Fluc mRNA by the viral polymerase consisting of L and P. The reporter mRNA is translated into luciferase protein which, following cell lysis, is assayed by addition of luciferin.

The existing PIV5 MG system has several limitations which were addressed during this project. Firstly, the reporter gene was changed from CAT to Firefly luciferase (Fluc), which is more sensitive and easier to assay. Secondly, rodent BSRT7 cells are not an ideal model for a primate virus as potential host factors may differ between species; this is particularly relevant when studying the effects of P protein phosphorylation on polymerase activity because this is dependent upon host kinases. Therefore, the highly-transfectable human embryonic kidney 293 cell line was used instead of BSRT7, with co-transfection of a plasmid expressing codon-optimised T7 RNA polymerase. Finally, additional modifications were attempted including the insertion of a Hammerhead ribozyme sequence to generate an authentic 5' end to the T7

transcript, and the inclusion of an SV40 terminator sequence to reduce background Fluc levels. In the following sections, the amounts of the five plasmids (MG, T7, NP, P, L) are optimised for use in 293 cells and the system is initially used to test a range of P protein mutants.

The MG plasmid with the CAT reporter replaced by Fluc is named pSMG-Fluc. The MG plasmid with the SV40 terminator is named pSMG-Fluc.ter and is used for all the below experiments unless stated otherwise. In addition to the five MG system plasmids, all transfections include pCAT-LAC, a plasmid which expressed β -galactosidase, the level of which can be assayed by addition of ortho-nitrophenyl- β -galactoside and reading absorbance at 420nm. This is used to control for transfection efficiency as detailed in the methods section. All transfections were performed in 293 cells.

3.1.1 Titration of MG helper plasmids

Reporter gene expression in the MG system should be dependent upon the co-transfected helper plasmids. Signal in the absence of any one of the helper plasmids is therefore considered 'background' which is not representative of viral replication or transcription. Leaving out any one of pCAGGS-NP, pCAGGS-P, pCAGGS-L, or pCAGGSV5-T7 resulted in a reduction of Fluc signal compared to when all plasmids were present in 293 cells (figure 3.2D). The signal/background ratio (S/B ratio) can then be calculated to give a measure of viral polymerase-dependent Fluc signal. From this point on, the negative control from which the S/B ratio is calculated consists of the complete MG system minus the plasmid expressing L.

As the viral enzyme responsible for RNA synthesis, the amount of L plasmid present is critical for MG system activity. Titration of transfected pCAGGS-L shows a dose-dependent increase in Fluc signal when all other MG components are kept constant (figure 3.2C). This provides further confirmation that the Fluc signal is a valid readout of viral RNA synthesis. Despite L being the least-transcribed gene during infection⁵⁰, a large amount of pCAGGS-L (500ng per well of a 6-well plate) was chosen to be used in the MG system based on these data because maximisation of the virus-specific Fluc signal makes the system more useful and because expressing L from a plasmid removes the likely limitation of having to transcribe a very large gene from the viral genome.

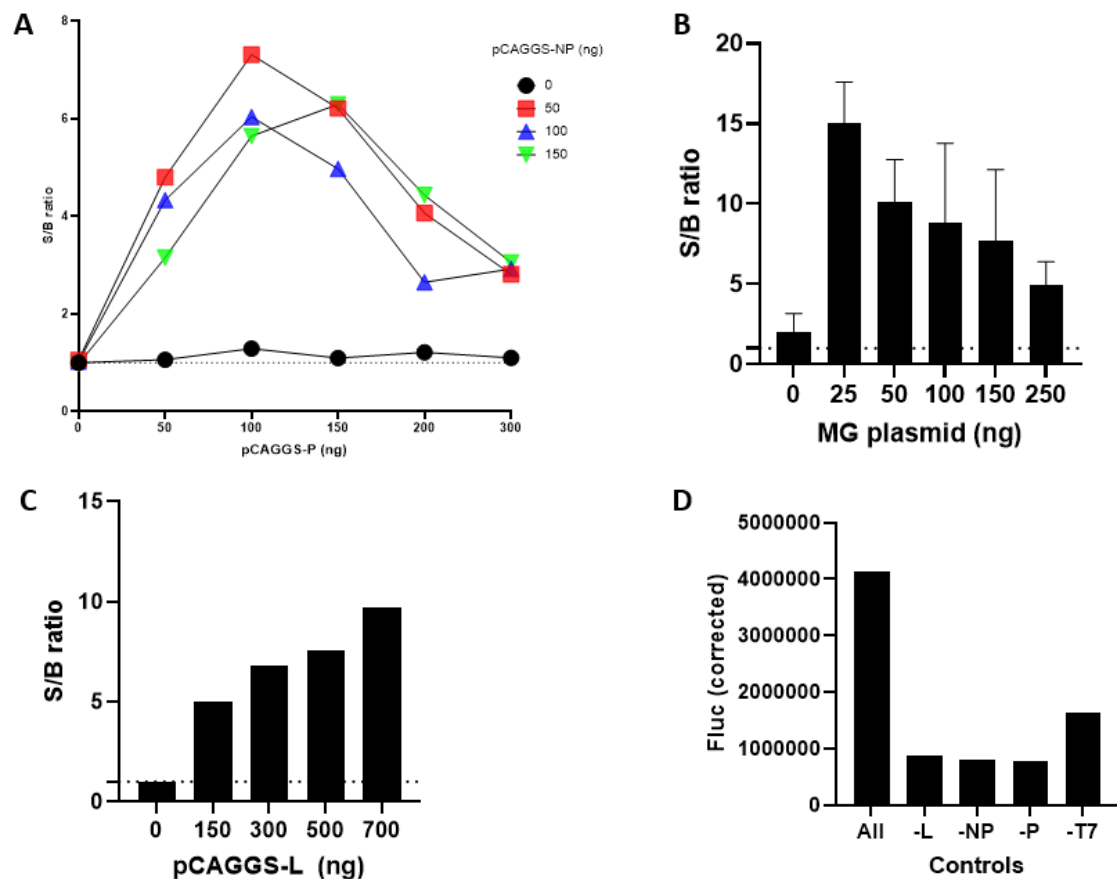


Figure 3.2 - Optimisation of MG helper virus plasmid amounts in 293 cells. **A)** The amounts of pCAGGS-NP and pCAGGS-P(W3A) were co-titrated while keeping all other plasmid amounts constant (500ng pCAGGS-L, 500ng pCAGGSV5-T7pol, 50ng pSMG-Fluc). **B)** The amount of pSMG-FLUC was varied while keeping all other plasmid amounts constant (100ng each of pCAGGS-NP and pCAGGS-P(W3A), 500ng pCAGGS-L, and pCAGGSV5-T7pol). **C)** The amount of pCAGGS-L was varied while keeping all other plasmid amounts constant (100ng each of pCAGGS-NP and pCAGGS-P(W3A), 500ng pCAGGSV5-T7pol, and 50ng pSMG-Fluc). **D)** Example of Fluc signal when leaving out any one of the helper plasmids. For **A-C**, a dotted line marks a S/B ratio of 1 on the y-axis below which there is no virus-specific reporter signal. For **B**, error bars are SEM and data are an average of at least three independent replicates. pCAT-LAC (expressing β -galactosidase) was co-transfected in all experiments to control for transfection efficiency, as detailed in the methods section.

In contrast to L which increases signal in a dose-dependent manner, levels of NP and P must be at an optimal ratio for formation of complexes between the viral proteins and regulation of the switch between viral transcription and replication^{31,32}. Therefore, the amounts of pCAGGS-NP and pCAGGS-P were co-titrated (figure 3.2A) with all other MG system components kept constant. For transfection of a single well of a 6-well plate, optimal S/B ratio was achieved with 100ng of pCAGGS-P and either 50ng or 100ng of pCAGGS-NP, indicating that an approximately 1:1 ratio of these two proteins is optimal for the MG system. The greater the disturbance of this ratio (e.g. 300ng pCAGGS-P and 50ng pCAGGS-NP), the lower the S/B ratio. Based on these data, it was decided to proceed using a 1:1 ratio of 100ng each of these plasmids per well of a 6-well plate. A lower ratio of NP to P was avoided because it would be less representative of viral gene expression during infection which produces more NP than P⁵⁰. However, the shorter MG RNA

(1,920 nt versus 15,246 nt for the whole virus) likely reduces the amount of NP required for encapsidation.

Finally, the MG plasmid was titrated to achieve an optimal S/B ratio (figure 3.2B). Lower amounts of transfected MG plasmid gave higher S/B ratios. This is probably because of reduced levels of non-L-specific generation of Fluc transcript, possibly due to cryptic transcription start sites in either the plasmid backbone or the viral sequences (leader or the NP 5'UTR). Despite this, by using a low amount of MG plasmid (i.e. 25ng per well of a 6-well plate), a good and reliable S/B ratio was achieved.

Based on the results of the helper plasmid titration data, the following amounts of plasmids were selected per well of a 6-well plate: 25ng pSMG-Fluc, 100ng pCAGGS-NP, 100ng pCAGGS-P, 500ng pCAGGS-L, and 500ng pCAGGSV5-T7. For assays in 12-well plates, the amounts of plasmids were scaled down by approximately 3-fold, as detailed in the methods section.

3.1.2 MG variants: SV40 terminator, Tr deletion, and Hammerhead ribozyme

Several variations were made to pSMG-Fluc in an attempt to improve its activity or utility, as shown in figure 3.3A. Firstly, an SV40 terminator sequence was introduced downstream of the T7 terminator in the reverse orientation. This should terminate any unwanted transcription being initiated from the plasmid backbone and increase S/B ratio by reducing background activity. However, this had minimal effect at reducing background and increasing S/B ratio (figure 3.3B) suggesting that there are cryptic start sites within the viral sequences or the T7 terminator sequence which contribute to Fluc signal in the absence of viral polymerase. Nevertheless, the SV40 terminator was included in the final MG plasmid used for the rest of this project.

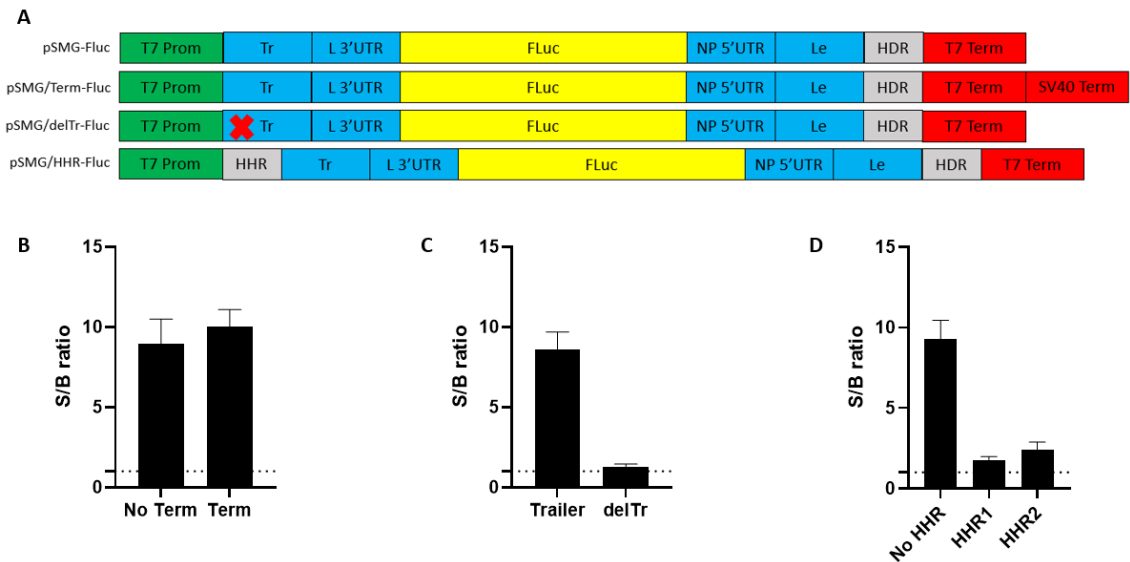


Figure 3.3 - Minigenome variants. **A)** Figure showing variations made to the MG construct: an SV40 terminator sequence introduced at the Leader end of the MG to reduce possible transcription initiation from the plasmid backbone; a 6nt deletion in the Trailer (delTr) which prevents viral replication while still allowing transcription; introduction of a hammerhead ribozyme (HHR) sequence downstream of the T7 promoter to generate an authentic 5' viral RNA sequence. **B)** Effect of introducing the SV40 terminator sequence. **C)** Functionality of the MG/delTr which is defective in replication. **D)** MG/HHR1 and MG/HHR2 incorporating different HHR sequences. For **B-D**, a dotted line marks a S/B ratio of 1 on the y-axis below which there is no virus-specific reporter signal. Error bars are SEM and data are an average of at least three independent replicates.

A second attempted modification made to the MG plasmid was the deletion of 6 nt in the Tr (delTr) to create a replication-defective MG. This modification has previously been used to specifically study the effects of V and P on transcription^{10,14,38}. However, no positive signal was seen in the conditions of these experiments (figure 3.3C). The published results using the delTr MG show a S/B ratio of less than 2 which means the majority of the reporter gene signal during the assay is not virus-specific; the very low activity of the delTr makes it an inappropriate measure of viral transcription and results using it are unlikely to be reliable. Interestingly, the low activity of replication-defective minigenomes is a common feature among *Mononegavirales*²¹⁵ even though the T7-transcribed MG RNA should be a good template for viral transcription provided that it is efficiently encapsidated by NP. One possibility not investigated here is that the HDV-generated 2'3'-cyclic phosphate (cP) at the 3' terminus greatly reduces the efficiency with which the viral polymerase is loaded onto the MG RNA. In this scenario, a small amount of replication of the MG RNA would be required to produce, via antigenome, a MG RNA with the correct 3'OH structure. A potential solution to this would be to in vitro transcribe the MG RNA, remove the cP by treatment with T4 PNK, and then transfect the RNA into cells along with the MG helper plasmids. Although laborious, a functioning replication-defective MG system would be useful to study viral transcription without the confounding factor of viral replication.

The final modification made to the MG plasmid was the introduction of a hammerhead ribozyme (HHR) sequence. A major limitation of using T7 polymerase is that the optimal T7 promoter sequence (TAATACGACTCACTATAGGG) requires the first three transcribed nucleotides (underlined) to be GGG²¹⁷. This is not an issue when generating mRNA by in vitro transcription, but for transcription of viral RNA (which begins ACC at both ends of the PIV5 genome), the non-viral GGG sequence disrupts the terminal regulatory sequences and causes the genome to break the 'rule of six'. The need for chance removal of this GGG by host exoribonucleases likely represents a major limiting factor in both MG system activity and in particular for rescue of full-length virus in paramyxovirus reverse genetics systems, including for PIV5, which can have rescue efficiencies as low as 1 in 10⁶ transfected cells^{213,218}.

A solution to this is to include a HHR sequence downstream of the T7 promoter which will self-cleave to generate an authentic viral 5' end, much like how the HDV ribozyme generates an authentic 3' end in the existing MG plasmid. This approach has yielded a greatly increased efficiency in the rescue of Nipah virus²¹⁹ and also been successfully used for MuV²¹⁸, MV²¹⁸, RSV²²⁰, and RABV²²¹. However, incorporation of a HHR ribozyme into the PIV5 MG plasmid completely abolished MG activity (figure 3.3D). This was true regardless of the length of the complementary target sequence (5 base pairs for HHR1, 10 base pairs for HHR2) suggesting that failure to form the expected RNA secondary structure was not to blame. However, differences in viral sequence between PIV5 and NiV could potentially either disrupt optimal HHR folding or create a T7-terminator like sequence. With regards to the latter point, the T7 terminator sequence consists of a poly-uridine tract downstream of a hairpin and a similar sequence is present in the PIV5 Tr downstream of the HHR structure (figure 3.4C).

Therefore, the cleavage efficiency of the HHR and the possibility of premature T7 termination were investigated. To test cleavage efficiency, RNA from cells transfected with pSMG-Fluc and pCAGGSV5-T7 was reverse-transcribed using a primer binding in the Fluc CDS and the cDNA was amplified by PCR with primers binding in either the Tr or HHR sequence so that the ratio of cleaved to uncleaved RNA could be determined (figure 3.4A). As shown in figure 3.4B, while using the MG plasmid as a control template produced a PCR product with the primer binding the HHR sequence, no PCR product was produced when using cDNA as template. This confirms that the HHR is cleaving correctly. However, the level of MG RNA generated by T7 could still be reduced by premature termination. To test this, the MG plasmid was digested with SacII to produce a template for T7 in vitro transcription which should produce a 94 nt product with no HHR or a 91 nt product plus a 52 nt cut-off with the HHR. If termination occurs with the HHR, then an additional 38 nt product will be present. As shown in figure 3.4D, no such product is produced which confirms that there is no premature T7 termination.

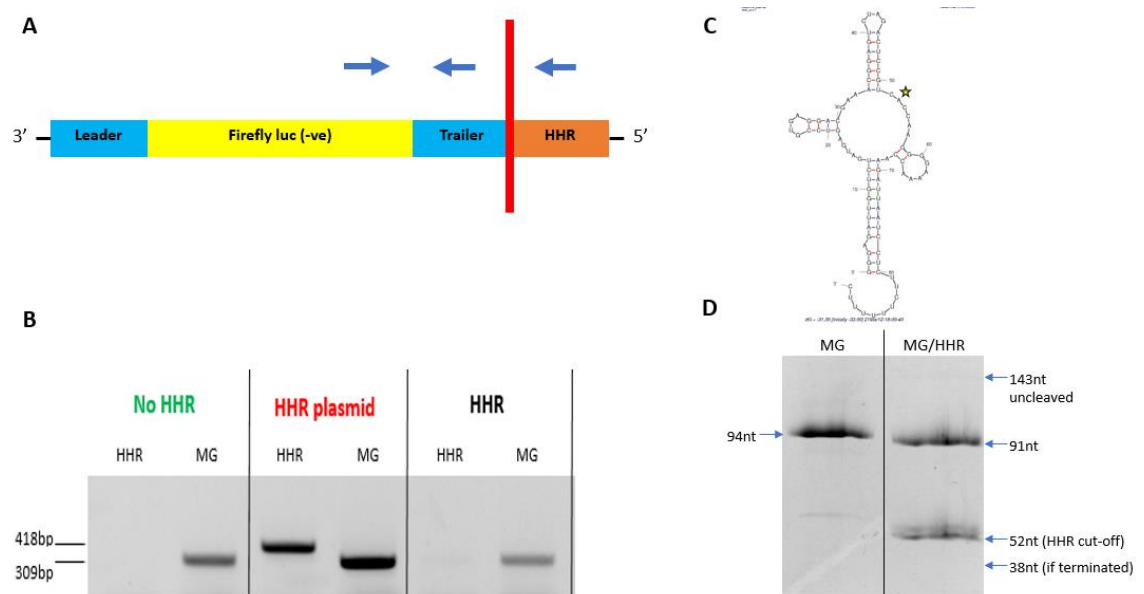


Figure 3.4 - Neither lack of HHR cleavage nor T7 termination explains failure of MG/HHR. A) Schematic showing primer binding sites for the RT-PCR ribozyme cleavage assay. **B)** RT-PCR HHR cleavage assay with RNA isolated from cells transfected with the MG system on the right. Negative RT-PCR control using a MG lacking the HHR is shown on the left ('No HHR'). Positive PCR control using the MG/HHR plasmid as template is shown in the middle ('HHR plasmid'). **C)** Predicted RNA secondary structure of the HHR (including the 5' GGG of the T7 promoter) followed by the PIV5 Tr sequence, with the first base of Tr shown by a yellow star. **D)** Denaturing gel electrophoresis analysis of truncated RNA products of T7-IVT using either the MG plasmid or the MG/HHR plasmid as template.

Together, these data show that the HHR is working as expected and fail to explain why inclusion of the ribozyme is deleterious to MG activity. One additional possibility is that the 5'-OH (rather than 5'-ppp) generated by HHR cleavage is somehow disadvantageous. Perhaps the non-authentic 5' structure disrupts either encapsidation by NP or run-off of the viral polymerase during replication, though these explanations would have to be specific to PIV5 because NiV and other viruses apparently tolerate the 5'OH. A working PIV5 T7/HHR system would be desirable to increase efficiency of virus rescue but this was not investigated further in this project.

3.1.3 Effect of P protein mutants on viral RNA synthesis

The published PIV5 MG system has mostly been used to study the effect of mutations in the phosphoprotein (P). P is an essential co-factor of the viral polymerase (L) and serves to mediated interactions between L and the RNA genome, which is encapsidated by NP. As its name suggests, the P protein is phosphorylated at multiple positions and many of these have an impact on its role in viral RNA synthesis, potentially by altering the nature of its interactions with L and NP. Other post-translational modifications (PTMs) are likely to include SUMOylation⁴⁰. A range of

these mutations, in addition to previously untested changes, were investigated using the optimised 293 MG system. 293 cells provide a more appropriate model for PIV5 than rodent BSRT7 cells because of the proposed impact of PTMs on the activity of P, which may operate in a host species-specific manner.

The most well-studied amino acid change in P is at position 157. The W3A reference strain has a serine at 157 while many naturally occurring strains (e.g. CPI+, which causes ‘kennel cough’ in dogs) have a phenylalanine at 157⁸⁴. The level of RNA synthesis by CPI+ is increased relative to W3A⁵⁰, and this has been mapped to the P protein with the S157F difference being largely responsible³⁸. The serine at 157 is phosphorylated³⁸, and the resulting ¹⁵⁵TSSpPI¹⁵⁹ motif (see figure 3.5, top) has been suggested to serve as a binding site for host polo-like kinase 1 (PLK1) which, according to the model, subsequently phosphorylates S308 of the P protein at a predicted PLK1 phosphorylation site²²², which results in reduced viral RNA synthesis by an unknown mechanism²²³. In this model, either disruption of the TSSpPI motif (e.g. by S157F or other changes) or preventing the phosphorylation of S308 (e.g. by S308A) abrogates the ability of PLK1 to downregulate polymerase activity. In support of the importance of these two locations in the P protein, substantial variation exists between strains at positions 155, 156, 157, and 159 (the putative TSSpPI motif)²²⁴. Single nucleotide polymorphisms (SNPs) have also been documented, mostly notably for strain H221 which has minor variants of S157F (4%) and K306N (5%)⁶⁹, the latter being proximal to the S308 residue as part of a conserved ³⁰⁶NDS³⁰⁸ motif (see figure 3.5, bottom). Furthermore, changes at 157 and 308 have been observed when attempting to rescue both PIV5 with C-terminally deleted V (VdelC) (Matthew Pickin, data unpublished) and with an NP unable to interact with M³⁰, with increased polymerase activity in both cases perhaps compensating for loss of full-length V or NP.

A thorough comparison of SNPs within the P gene has yet to be made in human cells. Therefore, a mixture of previously documented and untested naturally-occurring mutations were investigated using the 293 MG system and with P(W3A) as ‘wild-type’ reference. An alignment of the relevant P protein regions of selected PIV5 strains is shown in figure 3.5.

W3A	PRENPIATSSPIDFKRGAGIPA	169
AGS	PRENPIATSSPIDFKRGAGIPA	169
CPI+	PRENPIATSFPIIDFKRGAGIPA	169
DEN	PRENPIATSFPIIDFKRGAGIPA	169
H221	PRENPIATSSPIDFKRGAGIPA	169
D277	PRENPIATSSPTIDFKRGAGIPA	169
1168-1	PRENPIATNSPIDFKRGAGIPA	169
SER	PRENPIAPSSPIDFKRGAGIPA	169
KNU-11	PRENPIAPSSPIDFKRGAGIPA	169
	***** . *****	
W3A	PESFNDSFLTQS	313
AGS	PESFNDSFLTQS	313
CPI+	PESFNDSFLTQS	313
DEN	PESFNDSFLTQS	313
H221	PESFNDSFLTQS	313
D277	PESFNDSFLTQS	313
1168-1	PESFNDSFLTQS	313
SER	PESFNDSFLTQS	313
KNU-11	PESFNDSFLTQS	313
	**** : *****	

Figure 3.5 - Alignment of the TSSPI and NDS motifs in the P proteins of various PIV5 strains. Residues conserved across strains are shown in yellow; differences relative to P(W3A) are shown in blue. The serines at 157 and 308 are phosphorylated during infection^{223,224}. Alignment made with Clustal Omega. GenBank accession numbers for the PIV5 strains can be found in the methods (section 2.5).

The following changes were made in pCAGGS-P(W3A) around the TSSPI motif:

- T155P (naturally-occurring variant, e.g. in strains SER and KNU-11)
- S156G (occurred during serial passage of W3A, possibly due to selecting for rapid growth (Rick Randall, data unpublished))
- S156I (occurred during attempted rescue of VdelC (Mathew Pickin, data unpublished, and¹⁷))
- S157F (naturally-occurring variant, e.g. in CPI+^{81,84}, known to increase polymerase activity³⁸)
- I159T (naturally-occurring variant, e.g. in strain D277)

Additionally, two changes at the NDS sequence of the second phosphorylation site, S308, were made:

- N306K (naturally-occurring variant in strain H221⁶⁹)
- S308A (probable PLK1 phosphorylation site, previously documented²²³ and occurring during rescue of PIV5 with truncated NP (S308N)³⁰)

These changes were tested in the 293 MG system and compared to the activity level of P(W3A) (figure 3.6A). All changes were found to increase MG activity relative to P(W3A). Any disruption of the TSSpPI motif was sufficient to produce a similar increase to that seen with S157F, supporting the idea that this motif is critical for regulation of P protein activity. This could provide an explanation for why strains with S157 may still have high levels of RNA synthesis,

such as D277 (I159T) and 1168-1 (S156N). Interestingly, the most active P protein tested was T155P, as found in strains SER and KNU-11. Incorporation of a proline at this site may therefore change the protein structure in a way that favours a highly-active P protein. In addition to the TSSpPI motif, changes in the NDS sequence at positions 306 and 308 also resulted in an increase in MG activity. Similar to the comparison between P155 and F157, a change proximal to the putative phosphorylated residue (K306) produced a greater increase than changing the serine (A308). These data support a model in which sequential phosphorylations of the P protein regulate its activity and that the TSSpPI and NDS motifs are critical for this.

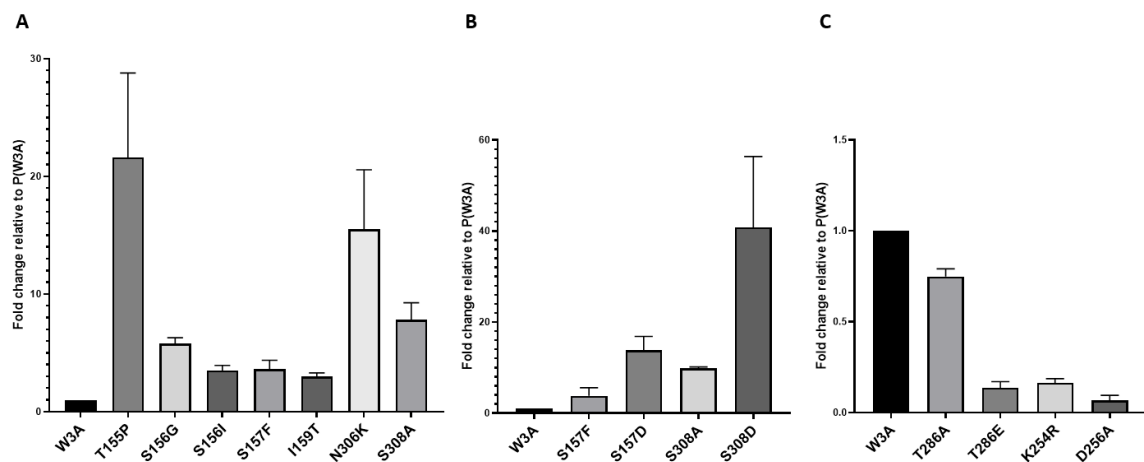


Figure 3.6 - P mutations tested with the MG system. A) A range of mutations relative to P(W3A) which are found either in naturally-occurring strains or have been identified during viral rescue experiments. **B)** The effect of introducing a phosphomimetic residue (aspartic acid, D) at positions 157 and 308 of P. **C)** Previously identified sites of probable PTM (phosphorylation at T286 and SUMOylation at K254) in P which have a negative effect on activity, as well as a mutation of the putative SUMOylation site (D256A). For **A-C**, the activity of each P protein is shown as fold change relative to the activity of P(W3A). Error bars are SEM and data are an average of at least three independent replicates.

Notably, the naturally-occurring variants at these locations are encoded by codons which vary by a single nucleotide. For example: S157 (TCC) and F157 (TTC), and T155 (ACC) and P155 (CCC). This could potentially provide a mechanism for PIV5 to switch between a high-activity state in which replication is favoured and transmission is more likely to occur due to increased disease severity (for example, kennel cough in dogs^{70,71}), and a low-activity state in which persistent infection is promoted by downregulating viral RNA synthesis and protein production. In support of this hypothesis, minor SNP variants of strain H221 have been detected (S157F and K306N) which show fluctuation between ‘high activity’ and ‘low activity’ residues in P. This idea is expanded upon in our recent publication²²⁴ which incorporates the MG data presented here as well as in vivo data from infections of mice. Most identified strains of PIV5 have ‘high-activity’ P proteins^{69,224} which is likely due to these strains causing disease and therefore being more likely

to be isolated. It would be interesting to obtain sequence data from subclinical infections of PIV5 to confirm the hypothesis that changes in P can promote persistent infection in vivo.

To test the idea that phosphorylation at S157 and/or S308 of P is required to downregulate RNA synthesis, the changes S157D and S308D were also generated and tested with the MG system. Aspartic acid has a similar structure to phosphoserine and is considered to be a 'phosphomimetic'; D157 and D308 would therefore be expected to serve as constitutively phosphorylated serine residues and, according to the PLK1 model, have MG activity as low as or lower than that of S157 and S308. Surprisingly, the opposite was found (figure 3.6B) with aspartic acid at both positions increasing activity relative to serine. This would suggest that properties of phosphoserine other than the negative charge are required for PLK1 binding and that the S157D and S308D changes simply disrupt the putative PLK1 binding site. However, one report attempting the same approach (albeit with glutamic acid rather than aspartic acid) with another protein phosphorylated by PLK1 found that changing the phosphorylated serine to an alanine abrogated PLK1 binding, but that changing to a phosphomimetic residue did not²²⁵. PLK1 may play an important role in the regulation of P protein activity, but clearly the mechanism by which it operates remains to be fully elucidated.

Finally, several other changes were made in P which have been found to have negative effects of MG activity in the BSRT7 MG system. They are the following:

- T286A (prevents phosphorylation of T286, required for full P functionality³⁹)
- T286E (prevents phosphorylation of T286, required for full P functionality³⁹)
- K254R (prevents SUMOylation of K254, required for full P functionality⁴⁰)
- D256A (disrupts the predicted consensus SUMOylation site; previously untested)

T286 has previously been shown to be phosphorylated³⁹. In contrast to S157, phosphorylation at this position increases polymerase activity and this was confirmed in the 293 MG system (figure 3.6C). K254 is a probable site of SUMOylation and this PTM has also been shown to be important for P protein activity⁴⁰, as confirmed in the 293 MG system (figure 3.6C). Lysine residues are often targets for multiple reversible PTMs and can serve as sites of protein function regulation²²⁶. Therefore, mutation of K254 could inadvertently prevent a PTM other than SUMOylation that would otherwise occur at some level. To avoid this issue, SUMOylation can be prevented by mutating the consensus SUMOylation motif (ψ -K-x-E/D, where ψ is a hydrophobic amino acid and x is any²²⁷) while preserving the modified lysine, for example by changing the E/D at position four to an alanine²²⁸. In PIV5 P, this sequence is 253-IKND-256 so the mutation D256A was made and tested in the MG system (figure 3.6C). This change was found to have a similarly severe effect on MG activity as K254R. While an effect not specific to SUMOylation can't be excluded based on these data, the D256A mutation could be a useful

starting point for further investigation of PTMs at K254, but this was not pursued any further in this project.

To confirm expression of the mutated P proteins investigated in this section, each pCAGGS-P plasmid was transfected into 293 cells and the proteins detected by western blot with a mouse anti-V5 antibody (figure 3.7). The V5 epitope is present in the P protein at amino acids 95-108 so all the mutant P proteins should be detectable. P migrates at an apparent size of approximately 51 kDa rather than its true size of 42 kDa due to multiple phosphorylations which affect its electrophoretic mobility. Varying levels of phosphorylation results in a 'laddering' pattern of multiple bands below the main band.

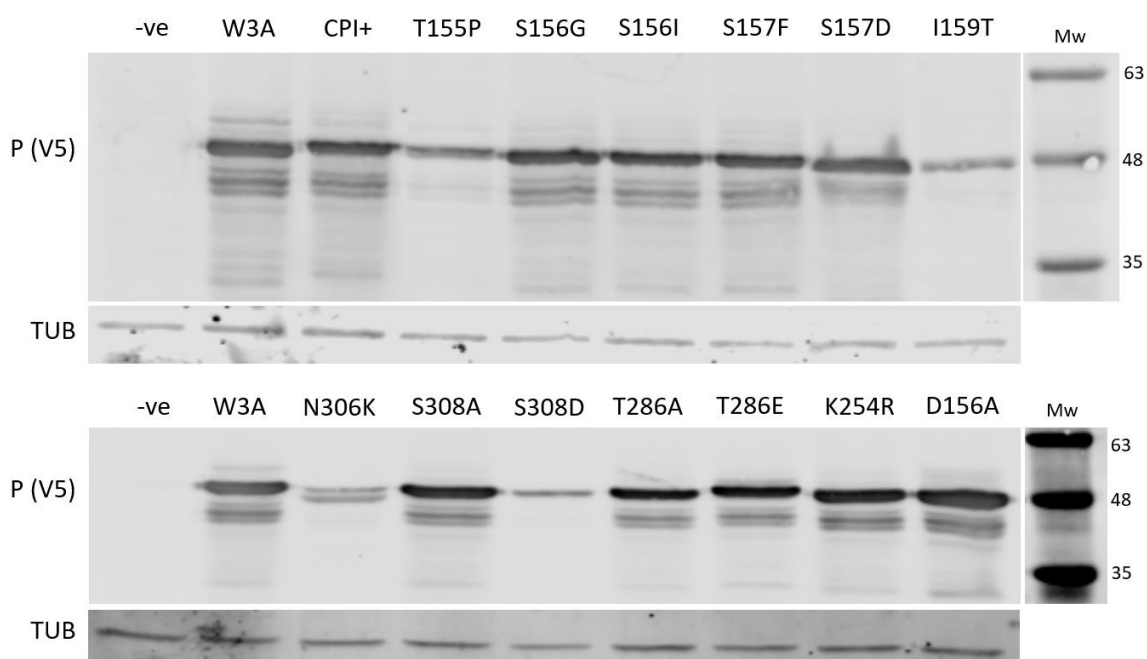


Figure 3.7 - Western blot of positive and negative P mutants. 293 cells in 12-well plates were transfected with 200ng of pCAGGS-P plasmid per well and cells lysed at 30h post-transfection. Protein extracts were separated by SDS-PAGE and proteins detected by western blot with mouse anti-V5 and rabbit anti-tubulin. The 14-aa V5 epitope is present in PIV5 P at positions 95-108. Molecular weight markers (Mw) are labelled in kDa.

Unexpectedly, the level of protein varied greatly between mutants. In particular, T155, I159T, N306K, and S308D were expressed at a much lower level than P(W3A). Paradoxically, three of these poorly-expressed P proteins (T155P, N306K, S308D) had the highest activity in the MG system (figure 3.6), suggesting a counter-intuitive mechanism in which instability of the P protein can promote its role in increasing transcription activity. The impact of NP:P ratio has already been established (for example, 3.2A), but the mechanism here is likely to be more complicated because I159T is poorly expressed but not as active than T155P. Another interesting result is that N306K appears to be post-translationally modified in a way that no other P mutant

is (for example, compare the double band of N306K to the single band of S308D). Lysines are targets for multiple PTMs, so K306 could be modified in a way that decreases protein stability and/or disrupts the previously discussed predicted PLK1 phosphorylation site. Although no mechanism has been elucidated for the effect of mutations in P on its role in transcription, it is clear that multiple amino acids and PTMs contribute to both its activity and stability.

3.1.4 Comparison of the P proteins of W3A and CPI+ in the MG system

As previously discussed, the differing rates of RNA synthesis between PIV5 strains has been mapped to changes within the P protein. In particular, the S157F difference between P(W3A) and P(CPI+) has been found to be responsible for the increased activity of the latter³⁸. There are 6 amino acid differences between P(W3A) and P(CPI+), as shown in figure 3.8, and a further 3 amino acid differences in the related P(CPI-) which result in CPI- V (L102P is in both P and V) being unable to bind STAT2 and target STAT1 for degradation⁸⁴. However, historical errors in the analysis of the CPI+ P sequence have led to confusion with some papers using a 'P(CPI+)' that contains T293 rather than K293⁸⁴ and G174/K184 rather than E174/R184. This misconception has blighted attempts to identify the residues responsible for phenotypic differences between P(W3A) and P(CPI+)^{38,229}. Using the correct P(CPI+) in the MG system, we have repeatedly noticed that it has a higher level of activity than P(W3A/S157F) (data not shown; Matthew Pickin, data not published). The previous study which concluded that S157F was solely responsible for the difference between these P proteins inadvertently used a P(CPI+) lacking the changes at 174, 184, and 293³⁸. Therefore, a thorough investigation of the relative impact of the 6 amino acid differences between P(W3A) and P(CPI+) was undertaken to explain these discrepancies.

W3A	MDPTDLSFSPDEINKLIETGLNTVEYFTSQQVTGTSSLGKNTIPPGVTGLTNAAEAKIQ	60
CPI+	MDPTDLSFSPDEINKLIETGLNTVEYFTSQQIIIGTSSLGKNTIPPGVTGLTNAAEAKIQ	60
CPI-	MDPTDLSFSPDEINKLIETGLNTVEHFTSQQIIIGTSSLGKNTIPPGVTGPLTNAAEAKIQ	60
	*****:*****:*****	
W3A	ESTNHQKGSVGGGAKPKKPRPKIAIVPADDKTVPKGPIPNPILGLDSTPSTQTVDLSGK	120
CPI+	ESTNHQKGSVGGGAKPKKPRPKIAIVPADDKTVPKGPIPNPILGLDSTPSTQTVDLSGK	120
CPI-	ESTNHQKGSVGGGAKPKKPRPKIAIVPADDKTVPKGPIPNPILGLDSTPSTQTVDLSGK	120

W3A	TLPSGSYKGVKLAKFGKENLMTRFIEEPRENPIATSPIDFKRGAGIPAGSIEGSTQSDG	180
CPI+	TLPSGSYKGVKLAKFGKENLMTRFIEEPRENPIATSPIDFKRGAGIPAGSIEESTQSDG	180
CPI-	TLPSGSYKGVKLAKFGKENLMTRFIEEPRENPIATSPIDFKRGAGIPAGSIEESTQSDG	180

W3A	WEMRSRLSGAIHPVLQSPQQGDLNALVTSVQSLALNVNEILNTRNLD SRMNQLETKV	240
CPI+	WEMRSRLSGAIHPVLQSPQQGDLNALVTSVQSLALNVNEILNTRNLD SRMNQLETKV	240
CPI-	WEMRSRLSGAIHPVLQSPQQGDLNALVTSVQSLALNVNEILNTRNLD SRMNQLETKV	240

W3A	DRILSSQSLIQTIKNDIVGLKAGMATLEGMITTVKIMDPGVPSNVTVEDVRKTLNHAHV	300
CPI+	DRILSSQSLIQTIKNDIVGLKAGMATLEGMITTVKIMDPGVPSNVTVEDVRKTLNHAHV	300
CPI-	DRILSSQSLIQTIKNDIVGLKAGMATLEGMITTVKIMDPGVPSNVTVEDVRKTLNHAHV	300

W3A	VPESFNDSFLTQSEDVISLDELARPTATSVKKIVRKVPPQKDLTGLKITLEQLAKDCISK	360
CPI+	VPESFNDSFLTQSEDVISLDELARPTATSVKKIVRKVPPQKDLTGLKITLEQLAKDCISK	360
CPI-	VPESFNDSFLTQSEDVISLDELARPTATSVKKIVRKVPPQKDLTGLKITLEQLAKDCISK	360

W3A	PKMREEYLLKINQASSEAQLIDLKKAIRSAI	392
CPI+	PKMREEYLLKINQASSEAQLIDLKKAIRSAI	392
CPI-	PKMREEYLLKINQASSEAQLIDLKKAIRSAI	392

Figure 3.8 - Alignment of the P proteins of PIV5 strains W3A, CPI+, and CPI-. Highlighted in yellow are residues in P(W3A) which differ in both P(CPI+) and P(CPI-). Highlighted in blue are residues in P(W3A) which differ in P(CPI-) but not P(CPI+). Alignment was made using Clustal Omega. GenBank accession numbers for the PIV5 strains can be found in the methods (section 2.5).

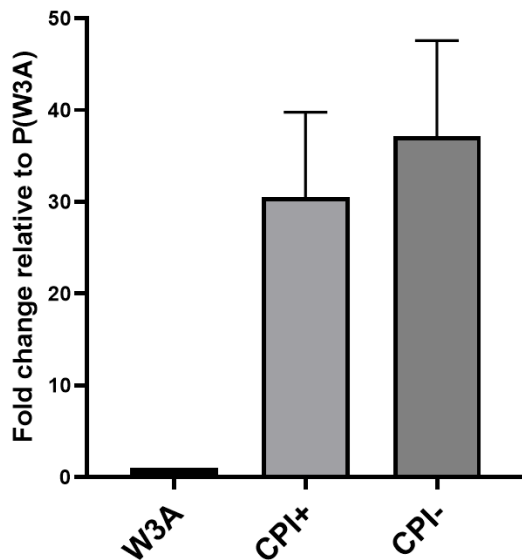


Figure 3.9 – Comparison of the P proteins of different PIV5 strains in the MG system. The activity of each P protein is shown as fold-change relative to the activity of P(W3A). Error bars are SEM and data are an average of at least three independent replicates.

Firstly, a comparison between P(W3A), P(CPI+), and P(CPI-) was made. Both CPI+ and CPI- were significantly more active than W3A (figure 3.9). Despite the additional 3 amino acid changes

between P(CPI+) and P(CPI-), there was no significant difference in activity. To determine the relative importance of the 6 amino acid differences between P(W3A) and P(CPI+), changes in P(W3A) were made in combination while treating V32I/T33I and G174E/K184R as single changes due to their proximity (to reduce to the total number of combinations for practical reasons). These chimaeric P proteins were then tested in the 293 MG system along with P(CPI+). As shown in figure 3.10, neither F157 alone nor any other single change is entirely responsible for the increased activity of P(CPI+). Most notable is the effect of T293K which, while having no major effect on its own, is able to increase the activity of F157, particularly when in combination with either V32I/T33I or G174E/K184R. The failure to include K293 in previous analyses³⁸ therefore likely explains the difference in activity seen between P(W3A/S157F) and P(CPI+). However, it is clear that maximal activity is only achieved when all changes are made in combination, showing that F157 likely interacts with the other locations to produce the phenotypic difference.

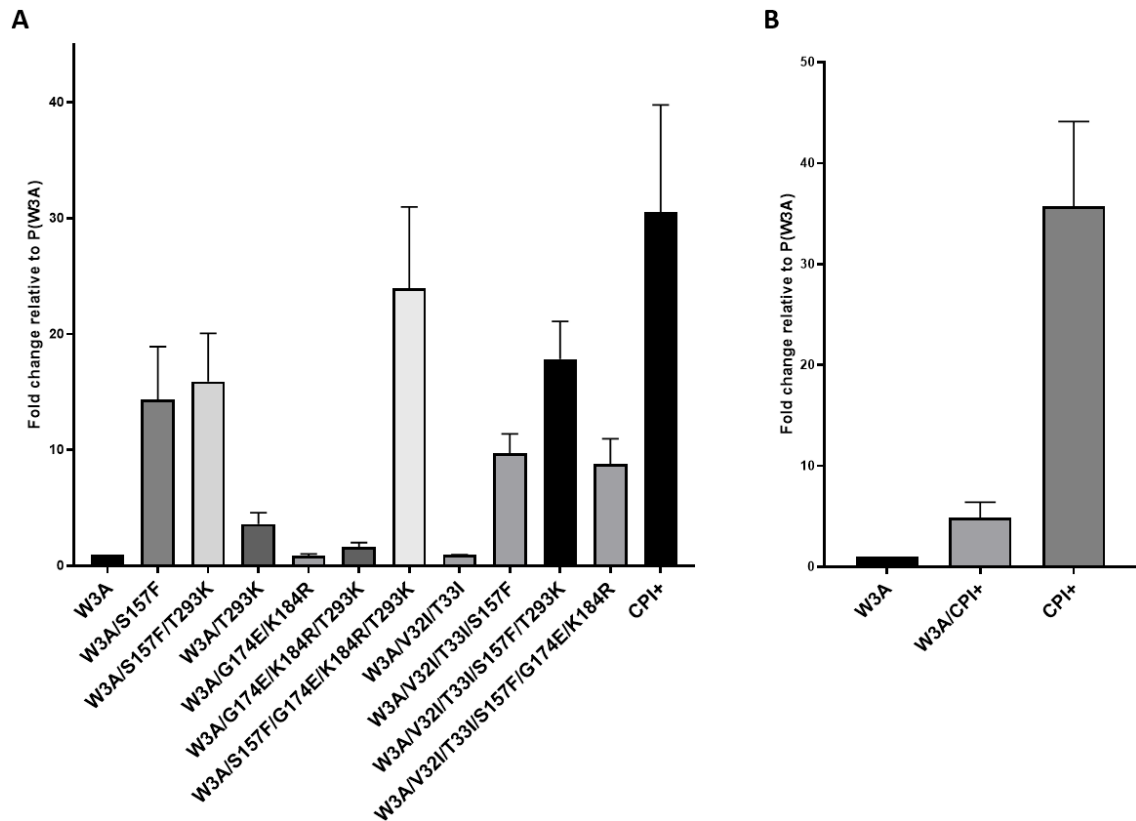


Figure 3.10 - The relative importance of amino acid differences between P(W3A) and P(CPI+). **A**) CPI+ residues were introduced into P(W3A) either singly (e.g. F157) or in combination and tested for MG activity relative to P(W3A) and P(CPI+). **B**) Co-transfection of P(W3A) and P(CPI+) while maintaining the same total amount of pCAGGS-P plasmid. For **A** and **B**, the activity of each P protein is shown as fold change relative to the activity of P(W3A). Error bars are SEM and data is an average of at least three independent replicates.

Finally, a co-transfection of P(W3A) and P(CPI+) was performed in which an equal amount of each pCAGGS plasmid was used while keeping the total amount of plasmid constant (figure 3.10B). When co-expressed, the MG activity level was found to be much nearer that of P(W3A) than P(CPI+), rather than the intermediate phenotype that one might expect. This suggests that P(W3A) disrupts the activity of P(CPI+), potentially by creating a rate-limiting step in transcription which negates the high activity of P(CPI+). Because the P protein forms a tetramer during RNA synthesis³⁶, it is possible that incorporation of a single 'low-activity' monomer of P is sufficient to reduce the rate of RNA synthesis.

As for the P mutants in the previous section, the expression level of each W3A/CPI+ chimaeric P protein was tested by transfection in 293 cells followed by western blot with an anti-V5 antibody. These P proteins are expressed from pCAGGSV5 and so are N-terminally V5-tagged in addition to the internal V5 epitope. This allows for detection of P(CPI-) which has an L102P change, disrupting the internal V5 epitope. Once again, the level of P protein varied greatly between mutants (figure 3.11). In contrast to the previous results, here low expression did not correlate with high MG activity as, for example, V32I/T33I and G174E/K184R were both poorly expressed and had low MG activity. However, V32I/T33I/S157F/T293K notably has high activity in the MG system despite being very poorly expressed relative to P(W3A). The lack of any obvious pattern in terms of either the effect of an amino acid change upon expression or the effect of expression upon activity makes it hard to draw any conclusions from these data. However, it is once again clear that the mechanism of how P activity is regulated by amino acid changes involves multiple residues and is likely determined by a combination of protein structure, PTMs, and protein stability.

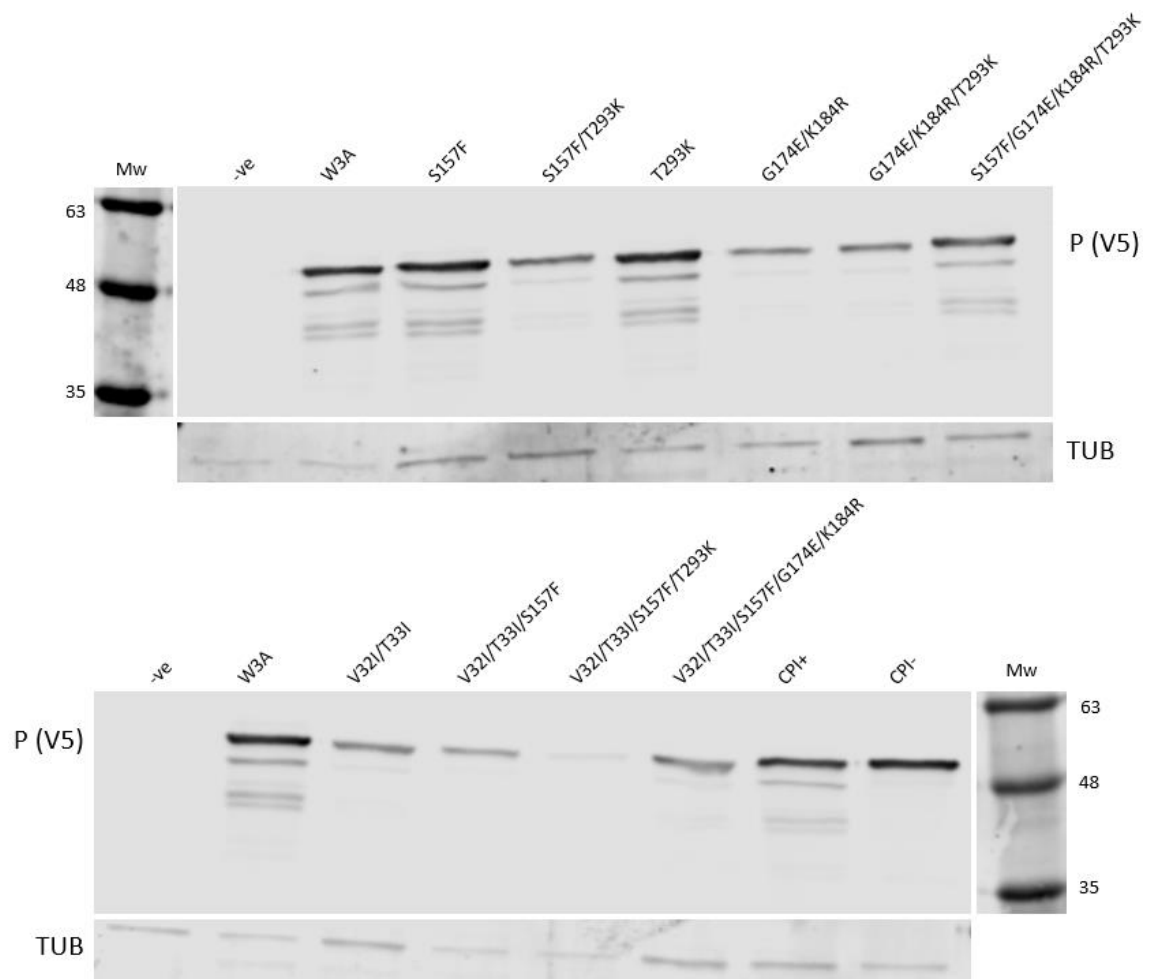


Figure 3.11 - Western blot of W3A/CPI+ chimaeric P mutants. 293 cells in 12-well plates were transfected with 200ng of pCAGGSV5-P plasmid per well and cells lysed at 30h post-transfection. Protein extracts were separated by SDS-PAGE and proteins detected by western blot with mouse anti-V5 and rabbit anti-tubulin. All proteins are N-terminally V5-tagged and the 14-aa V5 epitope is also present in PIV5 P at positions 95-108, except for in P(CPI-) due to the L102P change. Molecular weight markers (Mw) are labelled in kDa.

In summary, the PIV5 MG system provides a useful model to investigate the effect of P protein mutations on viral polymerase activity. The phenotypic difference in RNA synthesis between the PIV5 strains W3A and CPI+ has previously been shown to be primarily due to different rates of transcription⁵⁰; negative sense MG systems, such as that of EBOV, have been shown to primarily provide a readout of transcription^{215,230}, thus serving to emphasise the differences between, for example, P proteins with either S157 or F157. Despite the successful identification of important residues which regulate the activity level of P, a mechanism remains elusive. Regulation of P activity clearly involves phosphorylations (e.g. possibly by PLK1) and structural changes in the protein, but the specific step of viral transcription which is affected is not known. The role of P in mediating the interaction between L and the NP:RNA template implies that the P mutations either strengthen or weaken these interactions in a way that affects polymerase activity. The elongation speed of paramyxovirus polymerases is extremely slow (3 nt per second for MV²³¹

and just 1.7 nt per second for SeV⁵¹), with the rate-limiting step likely being the requirement for NP to be displaced from the genomic RNA template³³. Therefore, any effect of P mutations on this process could potentially have a large impact on the rate of viral transcription. Another possibility is that phosphorylation of P affects the formation of viral inclusion bodies, which serve as sites of replication and transcription. Such effects have been reported for the P protein of MV²³². The mechanisms which govern the activity of the P protein are an ongoing area of research.

3.2 Testing the sensitivity of paramyxoviruses to IFITs using the MG system

At this point, the focus of the project switched to the sensitivity of PIV5 to IFIT proteins. Due to the way in which IFIT1 acts (see introduction), it was hypothesised that the MG system used in the preceding sections may serve as a good model to begin investigating the sensitivity of PIV5 to IFIT1 and to address uncertainties from previous studies^{149,150}, the most recent of which was published in 2016 while the work in section 3.1 was ongoing.

Previous work from the Randall and Goodbourn groups identified Interferon-induced protein with tetratricopeptide repeats 1 (IFIT1) as the ISG primarily responsible for the sensitivity of PIV5 to human IFN^{149,150}. Although PIV5 V efficiently dismantles IFN signalling by targeting STAT1 for degradation^{7,8}, when infecting cells already in an antiviral state the virus will be exposed to ISGs. IFN-pre-treatment of cells reduces PIV5 plaque size and limits viral protein production but knockdown of IFIT1 was found to restore both plaque size and protein production to near-normal levels, thus implying that IFIT1 is the most important ISG during PIV5 infection¹⁴⁹. This sensitivity to IFIT1 was found to be unique to the *Rubulavirus* genus as other paramyxoviruses such as PIV3 are not sensitive¹⁵⁰.

IFIT1 is able to discriminate between 'self' and 'non-self' mRNA by the presence or absence of methylation at the 2' hydroxyl of the ribose of the first transcribed nucleotide^{130,178–180}. This 2'-O-methylation results in a 'cap1' structure, while mRNA unmethylated at the 2'-O position is a 'cap0' structure. Because the MG reporter gene mRNA is generated by the viral polymerase (figure 3.1), it potentially serves as a model for investigating sensitivity to ISGs which affect viral transcription and translation, including IFIT1. Having established and optimised the 293 MG system, it was decided to use it to further characterise the sensitivity of PIV5 to IFIT proteins.

3.2.1 Co-transfection of hIFIT1 in the MG system

An initial test of the suitability of the MG system to study sensitivity to IFIT1 was made by co-transfecting 293 cells with a plasmid expressing human IFIT1 (hIFIT1) along with the previously described MG plasmids in the following relative amounts per well of a 12-well plate: 8ng pSMG-Fluc.ter, 30ng pCAGGS-NP, 30ng pCAGGS-P, 150ng pCAGGS-L, 150ng pCAGGS-T7, and 250ng pEFV5-IFIT1. hIFIT1 reduced MG Fluc signal to about 45% of the control (figure 3.12A), showing that the MG system could be a viable model for inhibition by IFIT1.

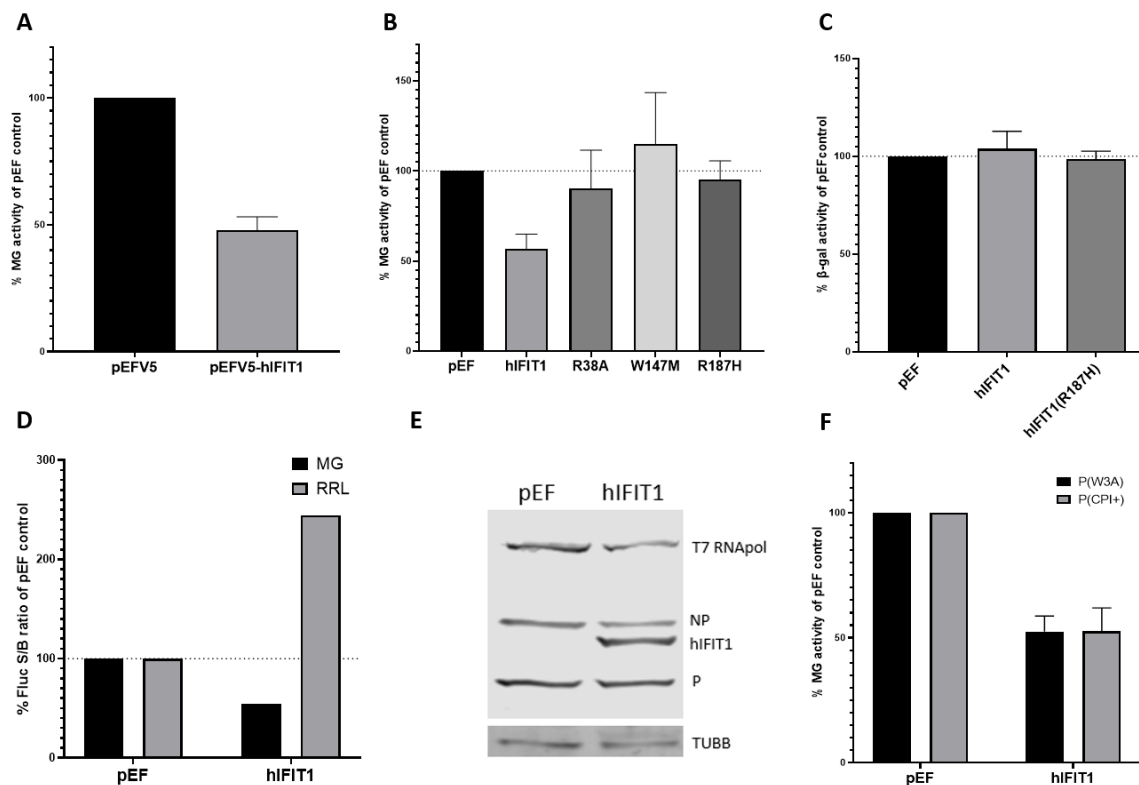


Figure 3.12 - hIFIT1 specifically inhibits the PIV5 MG. **A)** pEFV5-hIFIT1 was co-transfected with the PIV5 MG system plasmids into 293 cells in 6-well plates at the following plasmid amounts per well: 25ng pSMG-Fluc, 500ng pCAGGSV5-T7, 100ng pCAGGS-NP, pCAGGS-P, 500ng pCAGGS-L, and 750ng of either pEFV5 or pEFV5-hIFIT1. **B)** Mutations known to affect cap-binding of hIFIT1 were introduced and tested with the MG as in panel A. **C)** β -galactosidase activity in the MG assay, expressed from co-transfected pCAT-LAC plasmid in the presence or absence of co-transfected pEFV5-hIFIT1. **D)** The PIV5 MG assay transfection was performed as before with P(CPI+). Cells were harvested and split between MG assay and RNA isolation. The isolated RNA was quantified and an equal amount of each sample used for *in vitro* translation in the RRL system. Results are expressed as S/B ratio. **E)** 293 cells in 6-well plates were transfected with 25ng pSMG-Fluc, 500ng pCAGGSV5-T7, 100ng pCAGGSV5-NP, 100ng pCAGGS-P, and 750ng of either pEFV5 or pEFV5-hIFIT1. At 30h post-transfection cells were lysed and proteins analysed by SDS-PAGE followed by western blot for the V5 epitope. An equal amount of protein was loaded in each lane. **F)** Sensitivity to hIFIT1 was tested with the MG system either with P(W3A) or P(CPI+). For **A-C** and **F**, error bars are SEM and data are an average of at least three independent replicates.

A major potential issue with using the MG to study the effect of co-transfected ISGs is that the ISG may have an effect on helper gene expression rather than just reporter gene mRNA. Lower levels of NP, P, L, and/or T7 as a result of IFIT1 co-expression could also be responsible for the inhibitory effect of IFIT1 shown in figure 3.12A. This is a particular concern for hIFIT1, which has been reported to have a negative global effect on cellular translation by binding the translation initiation factor eIF3^{166,233,234}, as demonstrated by the inhibition by hIFIT1 of cap-independent translation from the HCV IRES, which requires eIF3²⁰³. Several approaches were taken to exclude this possible invalidation of the MG system as a model to investigate IFIT proteins. Firstly, hIFIT1 was co-expressed with NP, P, and T7 (L was excluded because of its extremely large size) and the levels of the proteins checked by western blot (figure 3.12E); co-expression of hIFIT1 had no major effect on protein levels. Secondly, mutations were made in hIFIT1 which are known to disrupt the structure of the RNA-binding pocket and prevent binding to the 5' end of RNA. These mutations (R38A, W147M, and R187H) have been previously characterised by mutagenesis informed by the crystal structure of hIFIT1 bound to RNA, and found to result in near-complete loss of cap0-binding¹⁸⁰. All three cap-binding mutations in hIFIT1 reduced its inhibition of the PIV5 MG (figure 3.12B) which strongly suggests that it is acting by binding to the reporter gene mRNA cap rather than causing a global reduction of translation by sequestering eIF3. Thirdly, the MG assays are performed with co-transfection of pCAT-LAC which expresses β -galactosidase, the level of which can be easily assayed (see previous section and methods section); hIFIT1 had no significant effect on β -galactosidase level (figure 3.12C). Fourthly, RNA was isolated from cells transfected with the MG system plasmids and either pEF or pEFV5-hIFIT1. If hIFIT1 is specifically inhibiting viral translation rather than helper gene expression, the level of MG Fluc mRNA should not be reduced and will be translatable once the IFIT1 protein has been removed during RNA isolation. The MG assay performed in parallel showed inhibition by hIFIT1, but in vitro translation of isolated mRNA showed no reduction of Fluc mRNA levels by hIFIT1 (figure 3.12D). This not only shows that the effect of hIFIT1 on the PIV5 MG is specific to viral translation, but also suggests that the functions of hIFIT1 do not include degradation of mRNA it is bound to (unless other ISGs are required for this to occur).

Finally, a comparison was made between P(W3A) and P(CPI+). Given P's role as a cofactor of the viral polymerase, there may be strain-specific differences in mRNA capping which could affect sensitivity to IFIT proteins. W3A is a lab-adapted PIV5 strain and sensitivity to IFIT1 has previously been shown to differ between wild type and lab-adapted strains of Venezuelan equine encephalitis virus²⁰⁸. Also, the large increase in the amount of mRNA produced by P(CPI+) relative to P(W3A) could reduce the effect of hIFIT1. However, there was no apparent difference in MG sensitivity to hIFIT1 between P proteins (figure 3.12F).

Together, these data show that the PIV5 MG is a valid system with which to study sensitivity to IFIT1. From this point on, it was decided to use P(CPI+) for experiments with the MG and IFIT proteins. There was no difference in sensitivity to hIFIT1 between P proteins, but the greatly increased MG signal seen with P(CPI+) provides a more robust assay than P(W3A) which often produces very low S/B ratios. 293 cells in which IFIT1 has been knocked out by CRISPR-Cas9 were made (see section 3.4.1 for details) so that any effect of basal levels of IFIT1 can be excluded. All of the following MG experiments with IFIT proteins are conducted in 293 IFIT1^{-/-} cells.

3.2.2 Sensitivity of the PIV5 MG to human, mouse, and chicken IFIT proteins

In addition to hIFIT1, the human IFIT family of proteins consists of hIFIT2, hIFIT3, hIFIT5, and hIFIT1B. Neither hIFIT2 nor hIFIT3 bind the 5' end of RNA¹⁷⁸ and instead have non-cap-binding effects. IFIT2 is involved in promoting apoptosis^{235,236}, is often downregulated in cancer cells^{237,238}, and can possibly bind internal AU-rich regions of RNA²³⁹. IFIT3 has antiproliferative effects^{240,241} and can interact with IFIT2 to modulate its apoptotic effect²⁴². hIFIT5 is well-characterised as a binder of 5' triphosphorylated (5'-ppp; 'uncapped') RNA²⁴³, a structure which is present on the 5' end of *Mononegavirales* genomes. Finally, hIFIT1B is not IFN-inducible due to the lack of ISREs in its promoter¹⁵⁸, is not able to be expressed as a recombinant protein¹⁷⁹, and is likely non-functional¹⁶¹. An alignment of human IFIT proteins is shown in figure 3.13.

An initial check was made of the effect of human IFIT proteins on MG helper gene expression by co-transfection with pCAGGS-P (figure 3.14A). As before, no effect was seen with hIFIT1 though it was poorly expressed relative to other IFITs. This may reflect that this western was performed at a later timepoint post-transfection than the western in figure 3.12E (48h versus 30h) and that hIFIT1 has a half-life of just 1.9h²⁴⁴. Interestingly, IFIT1(R187H), which is defective in binding to both cap0 and ppp RNA, was expressed at a much higher level and appeared to increase the level of PIV5 P, an effect also seen with the equivalent mutation in mIFIT1. It is not clear why the loss of RNA binding would promote cellular translation as no inhibition of translation is seen with the unchanged hIFIT1 protein. Similarly, hIFIT5 was also found to increase the expression of PIV5 P from pCAGGS. Unfortunately, for unknown reasons neither hIFIT2 nor hIFIT3 were detected despite being V5-tagged and having an effect on the MG (see figure 3.15). These two proteins have functions other than RNA binding, such as apoptosis, which are likely to affect helper gene expression. Finally, hIFIT1B had no effect on PIV5 P expression and was not detected by western blot, possibly because of its known instability. The RNA-binding human IFIT proteins

(hIFIT1 and hIFIT5) were also found to have no effect on β -galactosidase level (figure 3.14B). Together, these data show that any inhibition of the PIV5 MG by hIFIT1 or hIFIT5 is likely to be specific to viral processes, while any effect of hIFIT2 or hIFIT3 could be either antiviral or non-specific.

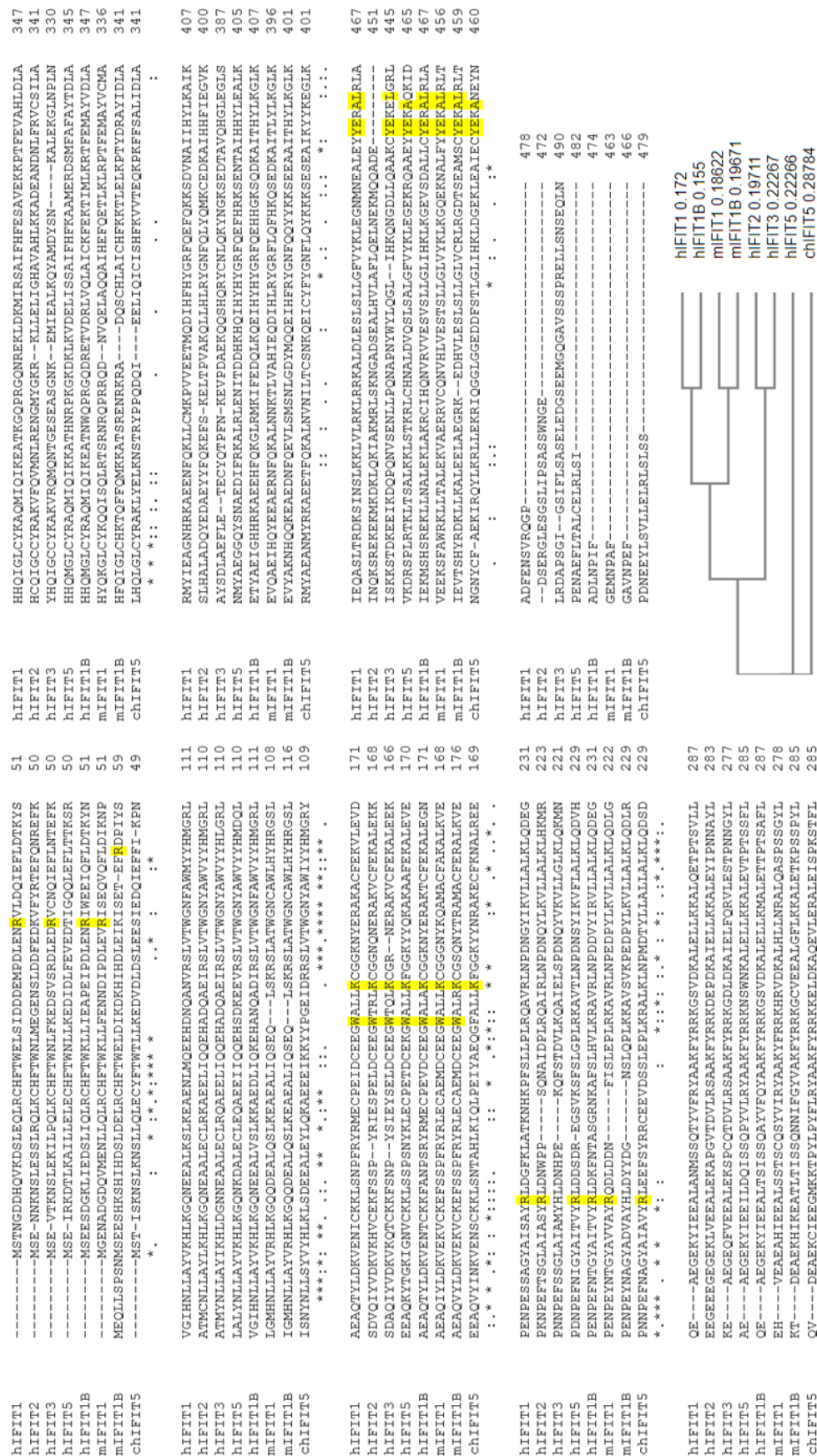


Figure 3.13 – Alignment of a selection of human, mouse, and chicken IFIT proteins investigated in this project. Conserved residues that have been mutated in this section are highlighted in yellow, as is the conserved C-terminal YxxxL motif. Also shown is a phylogenetic neighbour-joining tree. Alignment was made with Clustal Omega. GenBank reference number for each IFIT protein can be found in the methods (section 2.5).

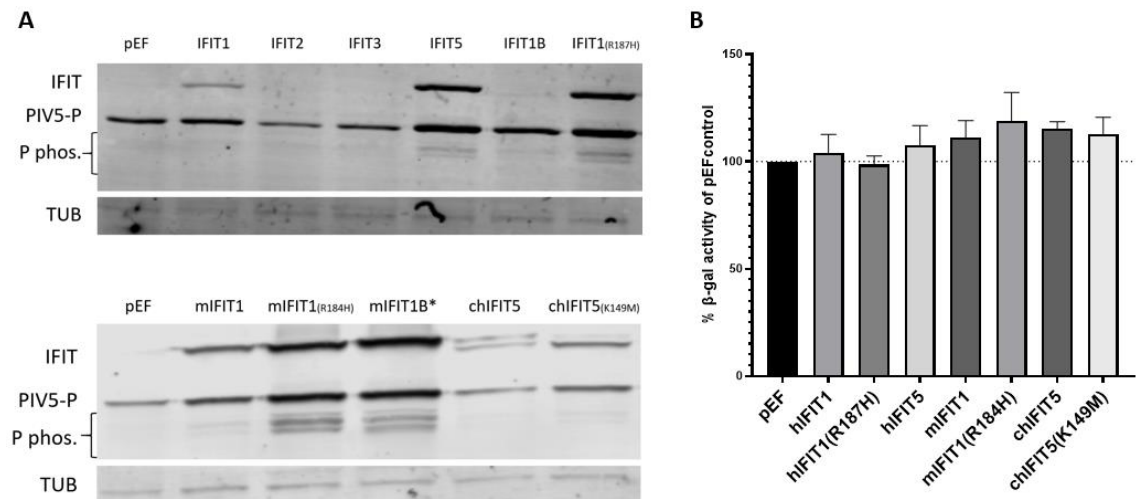


Figure 3.14 - The effect of IFITs on plasmid-expressed protein levels. A) pCAGGS-P (30ng per well of a 12-well plate) was co-transfected with the indicated pEFV5-IFIT plasmids (250ng). At 48h post-transfection cells were lysed and proteins analysed by SDS-PAGE followed by western blot with mouse anti-V5 and rabbit anti-tubulin. IFIT proteins are N-terminally V5-tagged; the V5 epitope is present within PIV5-P. Also indicated are the multiple faint bands of P which are due to phosphorylation ('P phos.'). An equal amount of protein was loaded in each lane. **B)** β-galactosidase activity in the MG assay, expressed from co-transfected pCAT-LAC plasmid in the presence or absence of selected co-transfected pEFV5-IFIT. Error bars are SEM and data are an average of at least three independent replicates

Each human IFIT was co-expressed in the PIV5 MG system as in the previous section. In contrast to hIFIT1, the other RNA-binding human IFIT (IFIT5) had no inhibitory effect and actually increased MG activity, possibly due its apparent positive effect on helper gene expression seen in figure 3.14A. hIFIT5 binds ppp RNA^{177,243}, a structure present at the 5' end of the PIV5 genome, but this requires an overhang of 3nt¹⁷⁷ and presumably the genomic terminus is shielded from hIFIT5 either due to RNA encapsidation by NP or by the formation of viral inclusion bodies. This, however, does not prevent hIFIT5 from inhibiting other *Mononegavirales* such as VSV and Influenza virus^{177,245}. The RNA-binding pocket of hIFIT5 cannot accommodate capped RNA¹⁷⁷, so the lack of an inhibitory effect of hIFIT5 suggests that the inhibition seen with hIFIT1 is due to mRNA cap-binding and not its low affinity for ppp RNA. One report suggests that hIFIT5 has a similar affinity for both ppp and cap0 RNA²⁴³, but this is not supported by the results with the PIV5 MG which likely produces cap0 mRNA (discussed at length in the following sections). Both IFIT2 and IFIT3 had a negative effect on the MG, but it is not possible to conclude whether they are acting upon viral or cellular processes. Finally, IFIT1B had no significant effect on the MG, consistent with reports that it is unstable and likely non-functional^{161,179}.

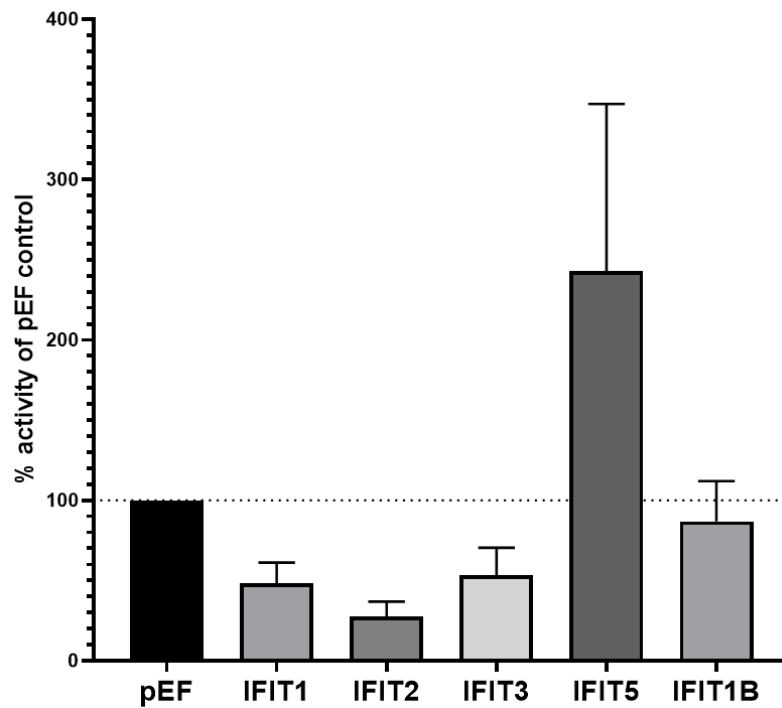


Figure 3.15 - Inhibition of the PIV5 MG by human IFIT proteins. The PIV5 MG plasmids (with P(CPI+)) were transfected into 293 IFIT1^{-/-} cells with or without pEF plasmids expressing the indicated IFIT. Error bars are SEM and data are an average of at least three independent replicates.

Rodents also encode multiple IFIT proteins. Confusingly, what is called mIFIT1 is a paralog of IFIT1 which diverged over 100 million years ago and likely has different, though similar, functions¹⁶¹. mIFIT1 is well-characterised as a cap0-binding protein with minimal affinity for other structures^{161,209,246}. In addition to mIFIT1, the mouse IFIT family includes mIFIT2, mIFIT3, mIFIT3B, mIFIT1B, and mIFIT1C. mIFIT1B and mIFIT1C are paralogs of mIFIT1, having arisen by gene duplication^{161,247}. mIFIT1B has recently been shown to exclusively bind cap1, though is much less IFN-inducible than mIFIT1²⁴⁷. The recent finding that hIFIT1 and mIFIT1 are not orthologous has led to a proposal to rename mIFIT1 as mIFIT1B¹⁶¹, but this creates an issue with the existing mIFIT1B and this nomenclature has not yet been adopted in the literature. Sendai virus naturally infects rodents, as do many other paramyxoviruses^{248,249}, and thus the antiviral activity of mouse IFIT proteins must be overcome by these viruses during infection.

mIFIT1 and mIFIT1B were selected for investigation as the known murine RNA-binding IFITs. Due to the lack of a commercially available cDNA clone, mIFIT1B was cloned from BALB/3T3 mouse cell cDNA. Upon sequencing, mIFIT1B was found to have multiple mutations resulting in changes at the N-terminus of the protein (figure 3.16C), including at position R54 which has previously been shown to be important for binding to cap1 RNA²⁴⁷. These changes likely render the protein non-functional and it is therefore referred to as mIFIT1B*. A significant portion of mouse mRNA is cap1 (as opposed to cap2)²⁵⁰ and mIFIT1B has been shown to inhibit host translation when overexpressed²⁴⁷. This apparent role in regulating host cell translation could have been selected

against during the immortalisation of BALB/3T3 cells and it would be interesting to investigate the sequence of mIFIT1B in other transformed mouse cell lines.

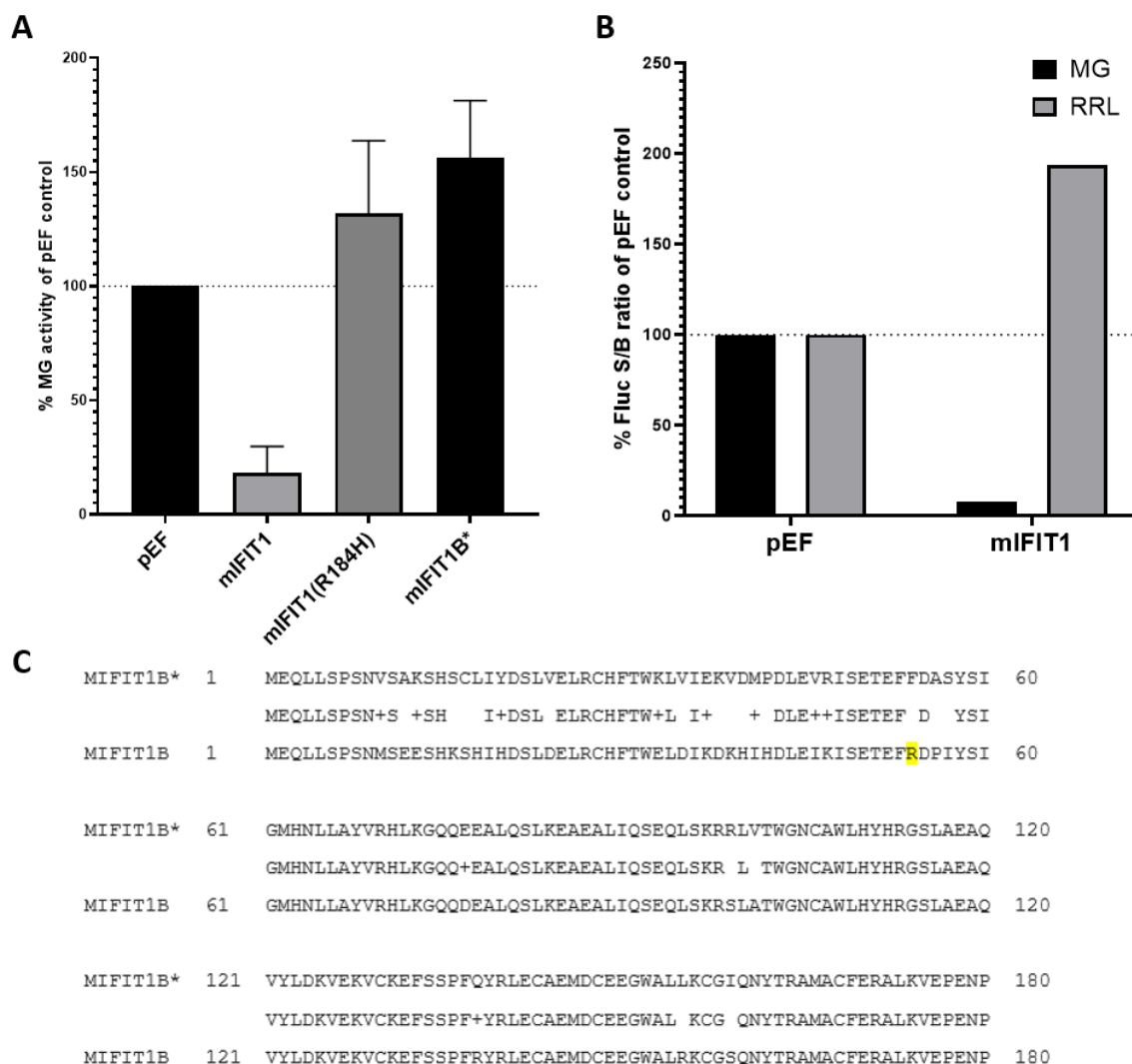


Figure 3.16 - Inhibition of the PIV5 MG by mouse IFIT proteins. **A)** The PIV5 MG plasmids (with P(CPI+)) were transfected into 293 IFIT1^{-/-} cells with or without pEF plasmids expressing mouse IFITs. **B)** The PIV5 MG assay transfection was performed as before with P(CPI+). Cells were harvested and split between MG assay and RNA isolation. The isolated RNA was quantified and an equal amount of each sample used for *in vitro* translation in the RRL system. Results are expressed as S/B ratio. **C)** mIFIT1B was amplified from mouse BALB/3T3 cells cDNA and cloned into pEF and sequenced. The encoded protein sequence (mIFIT1B*) was compared to the expected sequence of mIFIT1B, of which just the 180 N-terminal residues are shown. The difference at R54, a residue known to be important for cap-binding, is highlighted in yellow. For **A**, error bars are SEM and data are an average of at least three independent replicates.

Sensitivity of the PIV5 MG to mIFIT1 and mIFIT1B* was tested as before (figure 3.16A). The MG was highly sensitive to mIFIT1 with near-total (>90%) inhibition seen. This level of inhibition was greater than that seen with an equal amount of transfected pEFV5-hIFIT1 (figures 3.12 and 3.15). As mIFIT1 is a well-characterised cap0-binding protein, these data strongly suggest that the PIV5 MG is generating cap0 mRNA. The loss of mIFIT1 function seen when mutating the conserved

R184 residue mirrors the result seen with hIFIT1(R187H) and shows that the effect of mIFIT1 on the MG is specific to its cap-binding function. mIFIT1B* had no inhibitory effect on the MG which was to be expected regardless of whether this protein is functional as it binds cap1, not cap0. A functional mIFIT1B, however, might be expected to have an effect on cellular translation given its ability to bind cap1, but this was not seen when it was co-expressed with PIV5 P, perhaps further confirming that mIFIT1B* is non-functional due to the many N-terminal changes relative to mIFIT1B. mIFIT1 had a small positive effect on PIV5 P expression and no effect on β -galactosidase levels (figure 3.14), meaning that its inhibitory effect on the MG is likely specific to viral translation. As with hIFIT1(R187H), mIFIT1(R184H) increased PIV5 P expression relative to both pEF and mIFIT1 for unknown reasons. Further confirmation that the effect of mIFIT1 is specific to translation of the virally-generated Fluc mRNA is that following isolation of mRNA from cells transfected with the MG plasmids and pEFV5-mIFIT1, luciferase can be in vitro translated (figure 3.16B), as was previously shown for hIFIT1.

Birds, including chickens, have either lost or not acquired the full repertoire of IFIT genes found in mammalian genomes and – with a few exceptions – encode only an IFIT5-like gene^{160,161,245,251}. Chicken IFIT5 (chIFIT5) is the only avian IFIT that has been studied to any extent and a recent report has suggested that it shares at least some functions of hIFIT5 in that it is strongly induced by IFN²⁵², has some affinity for ppp-RNA²⁴⁵, and can inhibit replication of negative sense RNA viruses²⁴⁵. However, it is poorly characterised compared to human and mouse IFITs so warrants further investigation. Paramyxoviruses of the *Avulavirus* genus naturally infect a variety of avian species. The best-studied virus in this genus is Newcastle disease virus (NDV), which infects chickens.

The PIV5 MG is not inhibited by hIFIT5 (figure 3.15), so should chIFIT5 be a similar protein, i.e. one which solely binds ppp-RNA, a similar result should be seen. However, as shown in figure 3.17, chIFIT5 strongly inhibits the PIV5 MG to an extent greater than hIFIT1. This could be explained by chIFIT5 either having different functions to hIFIT5 or chIFIT5 causing cell toxicity or global translation arrest. To check the latter possibility, chIFIT5 was co-transfected with pCAGGS-P as before to check for any effect on the level of P. As shown in figure 3.14A, there was no significant effect on P expression (nor β -galactosidase levels in panel B), though chIFIT5 did appear as two products, possibly due to an unknown post-translational modification. Given that this is an avian protein being expressed in mammalian cells, the relevance of this is not clear and was not investigated further. Because chIFIT5 has no major effect on helper protein levels and hIFIT5 (which binds ppp-RNA) does not inhibit the PIV5 MG, it is probable that chIFIT5 is able to bind capped mRNA to specifically inhibit viral translation. This property of chIFIT5 has

not been previously documented and will be further investigated both later in this chapter and in chapter 5.

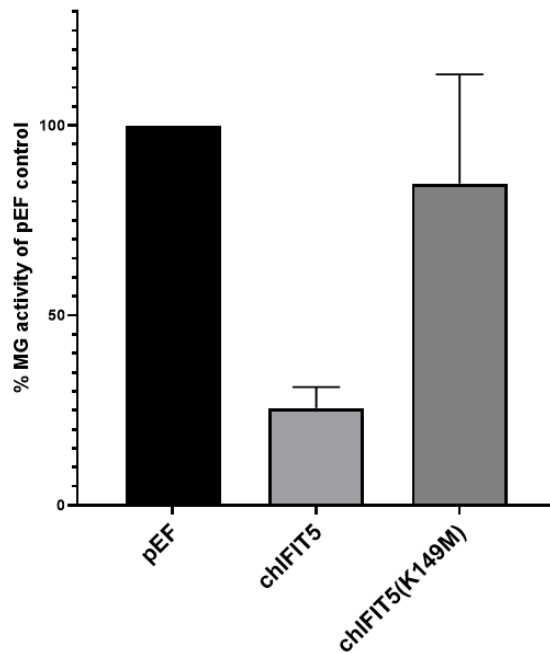


Figure 3.17 - Inhibition of the PIV5 MG by chicken IFIT5. The PIV5 MG plasmids (with P(CPI+)) were transfected into 293 IFIT1^{-/-} cells with or without pEF plasmids expressing chIFIT5 or chIFIT5(K149M). Error bars are SEM and data are an average of at least three independent replicates.

A K150M mutation in hIFIT5 abolishes its affinity for ppp-RNA^{177,243}, and the one report suggesting that hIFIT5 can bind cap0 also found that K150M cannot bind cap0²⁴³. Alignment of chIFIT5 with hIFIT5 (figure 3.13) shows that this lysine (K149 in chIFIT5) is conserved, as it is across multiple IFITs of different species. Therefore, the equivalent mutation (K149M) was made in chIFIT5 to check whether its inhibitory effect on the PIV5 MG is dependent upon binding to the 5' end of RNA. The K149M change greatly reduced chIFIT5's inhibitory effect (figure 3.17), presumably due to loss of 5' RNA binding. Interestingly, the possible PTM seen on chIFIT5 by western blot was reduced by the K149M change (figure 3.14A) suggesting that K149 is either the modified residue or is involved in the modification. Also of note is the apparent small increase in P levels seen with chIFIT5(K149M) relative to chIFIT5, an effect also seen for hIFIT1(R187H) and mIFIT1(R184H).

3.2.3 The cooperative effect of hIFIT1 and hIFIT3

It is notable that at the same amount of plasmid used, mIFIT1 has a much stronger effect on the PIV5 MG than hIFIT1 does (compare figures 3.15 and 3.16A). hIFIT1 has previously been shown to interact with hIFIT2 and hIFIT3^{152,162,178}, both of which are also strongly induced by IFN but do not bind capped RNA on their own. Therefore, it was hypothesised that additional human IFIT proteins may be required to co-operate as part of the 'IFIT interactome' to achieve the maximal level of inhibition achieved by mIFIT1. The interaction between all combinations of these three proteins was confirmed by yeast-2-hybrid, a technique which assesses protein-protein interactions by fusing candidates to either the binding or activation domain of a yeast transcription factor and using gene expression of a reporter as a readout. Yeast growth was clearly visible even at high concentrations of 3-AT (figure 3.18A), which prevents false positive results from weak interactions, thus showing that the three IFIT proteins strongly interact with each other. This supports the well-established interactions between human IFITs 1-3 previously reported in mammalian cells.

To investigate the potential role of IFIT interactions on their antiviral activity, hIFIT1, hIFIT2, and hIFIT3 were co-transfected either alone or in combination in a 1:1:1 ratio (figure 3.18B). This experiment is complicated by the negative effect of IFIT2 and IFIT3 on the MG even in the absence of IFIT1, as documented in the previous section. Even at the lower amount of plasmid used here (83.33ng per well of a 12-well plate), IFIT2 and IFIT3 inhibit the MG. Both IFIT2 and in particular IFIT3 increased the inhibition seen with IFIT1 but the effect of each IFIT on its own means that it is not possible to determine whether these effects are additive or cooperative. The greatest level of inhibition of the MG was seen when all three IFITs were expressed together, but the increased inhibition upon addition was minimal compared to that achieved by IFIT1/IFIT3, which was comparable to that seen with mIFIT1 in the previous section and provides weak evidence for cooperation between human IFIT1 and IFIT3 being needed to achieve a maximal inhibitory effect on viral translation.

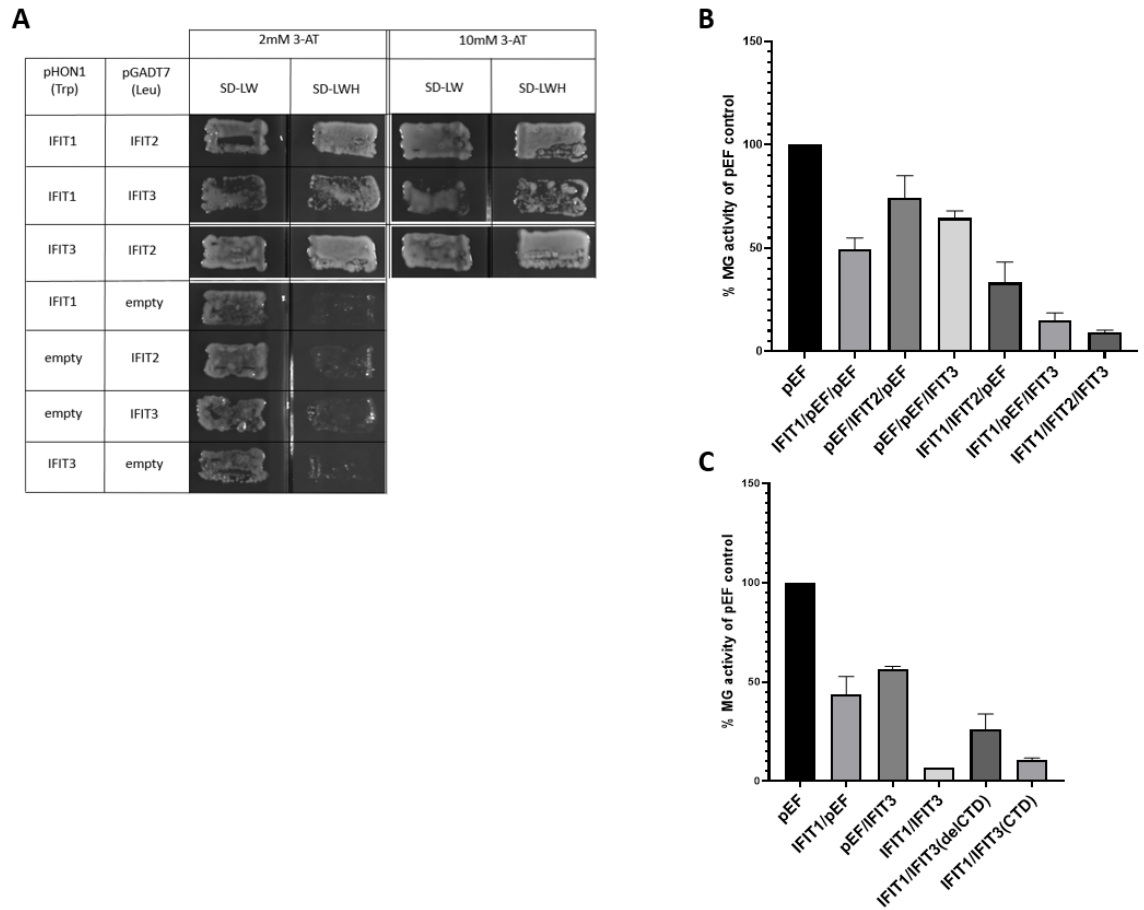


Figure 3.18 - Cooperation between hIFIT proteins. A) Yeast-2-hybrid to confirm interactions between hIFIT1, 2, and 3. Yeast strain PJ69-4a was transformed with the indicated IFIT expressed from either pHON1 (Trp) or pGADT7 (Leu) and plated on either SD –LW (Leu/Trp selection) or SD –LWH (Leu, His, Trp selection) selective plates at either 2mM or 10mM 3-AT. Growth on SD-LWH only occurs when the proteins interact. Controls with singly-expressed IFITs are also shown. **B)** Equal amounts (83.33ng per well of a 12-well plate) of hIFIT1, hIFIT2, and hIFIT3 were co-transfected either alone with empty pEF vector or with other IFITs and tested with the PIV5 MG system in 293 IFIT1^{-/-} cells as before. **C)** Same as for B, but with hIFIT1 and either hIFIT3, hIFIT3(delCTD) which has the C-terminal domain deleted, or hIFIT3(CTD) which is the C-terminal alone. For B and C, error bars are SEM and data are an average of at least two independent replicates.

Subsequent to these experiments being performed, two publications corroborated the cooperative role of hIFIT3 and determined the mechanism by which it achieves this function^{244,253}. hIFIT3 was found to both increase the half-life of hIFIT1 and to induce a conformational change which increases the affinity of hIFIT1 for cap0 specifically. This was dependent upon the C-terminal YxxxL motif (see figure 3.13), found in both hIFIT1 and hIFIT3, which facilitates dimerisation¹⁶², and the C-terminal 87 amino acids of hIFIT3 alone were sufficient to promote hIFIT1 cap0-binding²⁴⁴. To confirm that the effect of hIFIT3 in the PIV5 MG is due to the same mechanism, the CTD of hIFIT3 was deleted (hIFIT3(delCTD)) or expressed without the N-terminal 403 amino acids (hIFIT3(CTD)). As shown in figure 3.18C, the hIFIT3 CTD alone was sufficient to promote the inhibition of the PIV5 MG by hIFIT1, while the absence of the CTD partially abrogated this function of hIFIT3.

The role of interactions between IFIT proteins is an emerging field and has been recently reviewed¹⁶³. For example, mIFIT1C has been shown to promote translation inhibition by both mIFIT1 and mIFIT1C in a manner similar to that of hIFIT3 and hIFIT1²⁴⁷. Co-expression of the IFIT proteins of various species in MG systems could serve as a model to investigate such cooperative interactions.

3.2.4 Comparison of the sensitivity of PIV5, MV, and NDV to IFIT proteins

Sensitivity to IFIT1 has been reported to be specific to the *Rubulavirus* genus, with other paramyxoviruses including SeV, CDV, PIV3¹⁵⁰, as well as other *Mononegavirales* families¹⁹³, being insensitive. To both validate the results seen with the PIV5 MG and to further characterise human, mouse, and chicken IFIT proteins, we constructed MG reporters for Measles virus (MV, strain Edmonston) and Newcastle disease virus (NDV, strain LaSota). These MG systems follow the same design and protocol as has been optimised for the PIV5 MG and, importantly, the helper genes (NP, P, L, and T7pol) have all been cloned into the same vector (pCAGGS) and thus have the same promoter and 5'UTR. This allows for comparison between virus MGs as any effect of IFITs on helper gene expression via either mRNA cap-binding or global translation arrest would be the same.

As a mammalian non-*Rubulavirus* paramyxovirus, the MV MG would be expected to produce cap1 mRNA and therefore be insensitive to IFIT1, as suggested by previous results with SeV¹⁵⁰. In contrast, the sensitivity of avulaviruses such as NDV to IFIT1 has never been tested because these viruses do not infect mammals, while chickens lack a known cap-binding protein such as IFIT1¹⁶¹. The mRNA generated by the NDV polymerase has been reported to have a cap0 structure²⁵⁴ and would therefore be predicted to be sensitive to IFIT1. Together, these MG systems should allow for confirmation of the results seen with the PIV5 MG, with MV serving as a probable cap1 control that should be insensitive to IFIT1 and NDV serving as a cap0 control that should be sensitive to IFIT1.

The three MGs were tested for sensitivity to the RNA-binding IFIT proteins hIFIT1, hIFIT5, mIFIT1, and chIFIT5 along with the previously described mutants of hIFIT1, mIFIT1, and chIFIT5. As shown in figure 3.19, the MV MG was completely insensitive to the cap0-binding proteins hIFIT1 and mIFIT1, as well as the ppp-RNA-binding protein hIFIT5. Interestingly, these proteins all actually increased the signal for unknown reasons, in particular mIFIT1. This is perhaps reflective of the apparent increase in helper gene expression by mIFIT1 seen previously (figure 3.14A). The lack of a negative effect of hIFIT1 and mIFIT1 is to be expected given the assumption that MV

produces cap1 mRNA. However, chIFIT5 unexpectedly had a strong inhibitory effect, similar to its effect on the PIV5 MG. As discussed in the previous section, the lack of inhibition seen with hIFIT5 suggests that MV is not inhibited by ppp-RNA binding proteins and therefore chIFIT5 is likely acting by binding capped mRNA. Assuming that the MV MG is producing cap1 mRNA, this would mean that chIFIT5 can bind both cap0 (PIV5) and cap1 (MV) structures. Again, this will be further investigated in chapter 5.

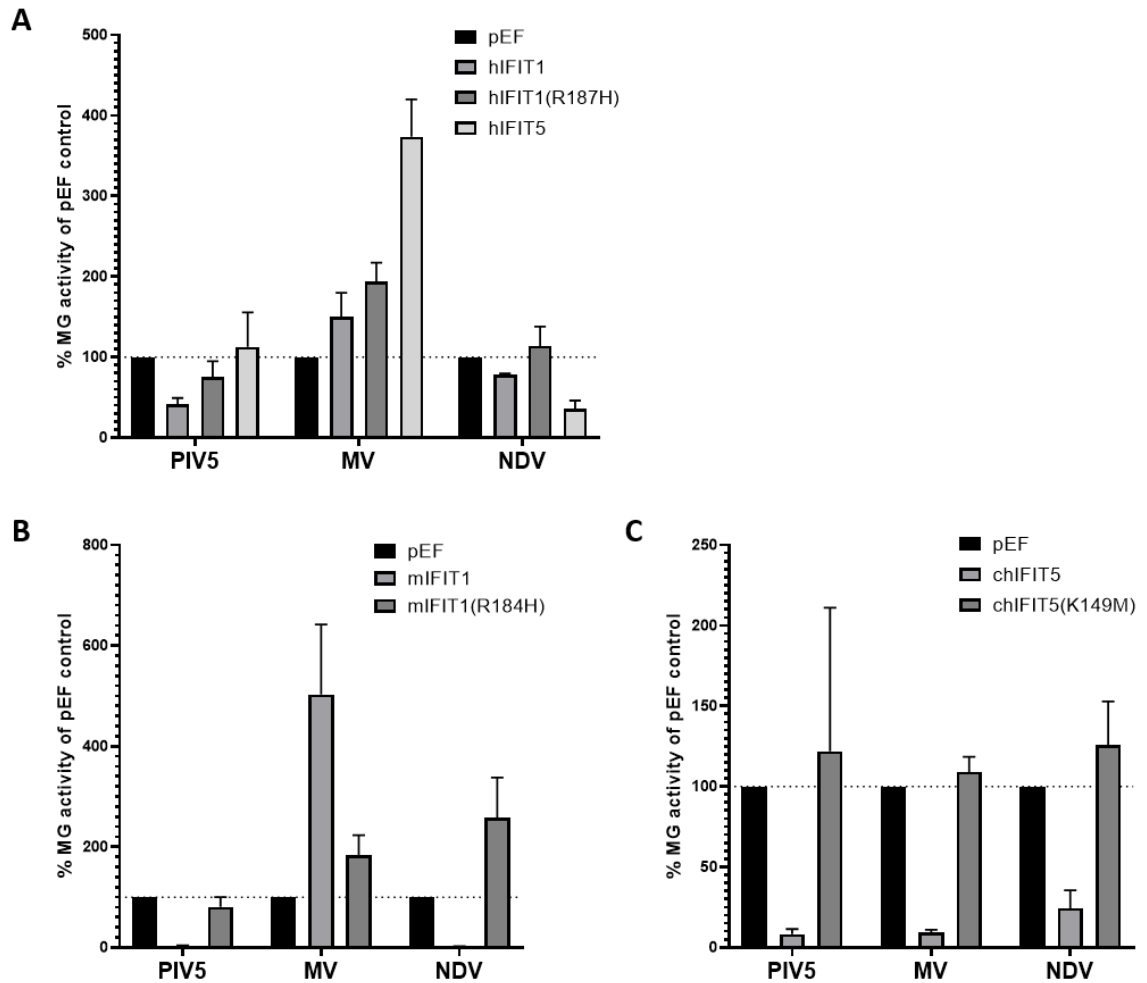


Figure 3.19 - The sensitivity of the PIV5, MV, and NDV MG systems to RNA-binding IFIT proteins. The MG system plasmids were transfected into 293 IFIT1^{-/-} cells as before, together with the indicated human (**A**), mouse (**B**), and chicken (**C**) IFITs expressed from pEF. The amount of each pEFV5-IFIT plasmid was kept constant at 250ng per well of a 12-well plate. Helper plasmid amounts were as follows: 8ng MG plasmid, 150ng pCAGGSV5-T7, 30ng pCAGGS-NP, 30ng pCAGGS-P, and 150ng pCAGGS-L. Error bars are SEM and data are an average of at least three independent replicates.

In contrast to MV, the NDV MG was highly sensitive to mIFIT1 (but not mIFIT1(R184H)), as was expected for a virus which likely produces cap0 mRNA²⁵⁴. However, NDV appears to be inhibited to a much lesser extent by hIFIT1 than PIV5 is. chIFIT5 inhibited the NDV MG in agreement with published data using the virus itself²⁴⁵. Santhakumar *et al.* concluded that the antiviral effect of

chlFIT5 was dependent upon its ability to bind ppp-RNA. However, given that chlFIT5 can apparently also bind cap0/1 mRNA (as implied by its inhibition of the MV MG), it is possible that chlFIT5 acts upon both NDV ppp-RNA and cap0 mRNA. The K149M mutation in chlFIT5 completely abrogated its effect on the MG, again confirming that its action is likely dependent upon binding to the 5' end of RNA.

The cap structure of PIV5, MV, and NDV transcripts can be inferred from the MG results presented above. Because mIFIT1 is known to bind only cap0 with high affinity, PIV5 and NDV are likely producing cap0 mRNA, whereas MV is likely producing cap1 mRNA. The recently published role of hIFIT3 in increasing the affinity of hIFIT1 for cap0 but not cap1 or ppp-RNA^{244,253} allows for further confirmation of this inference of cap structures. As in section 3.2.3, hIFIT1 and hIFIT3 were expressed alone or together in a 1:1 ratio while maintaining the same total amount of pEF plasmid (figure 3.20). As before, hIFIT1 alone had a negative effect on PIV5, a small effect on NDV, and no negative effect on MV, while hIFIT3 alone had a small effect on PIV5 and no effect on NDV or MV. Co-transfection of hIFIT1 and hIFIT3 produced a greatly increased inhibitory effect upon the PIV5 MG as well as a strong inhibition of NDV, despite NDV not being greatly affected by either protein alone. In contrast, MV remained completely insensitive to hIFIT1 despite the presence of hIFIT3. These data strongly suggest that both PIV5 and NDV produce cap0 mRNA, while MV produces cap1. It is not clear why NDV should be less sensitive to hIFIT1 than PIV5 is, nor why hIFIT3 has a negative effect on the PIV5 MG but not the MG of MV or NDV.

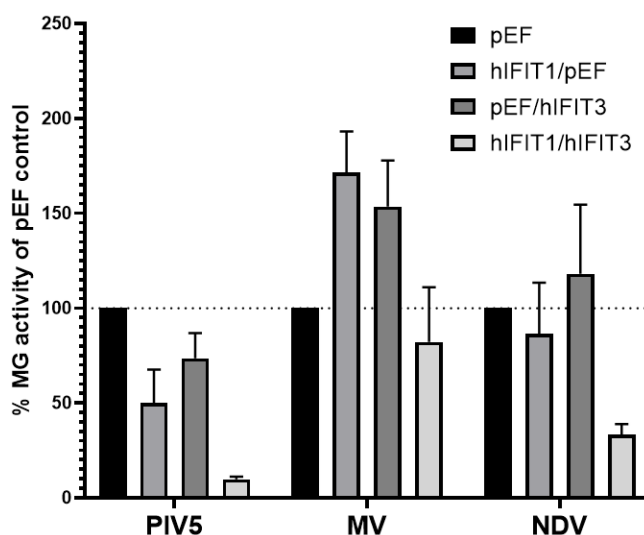


Figure 3.20 - The co-operative effect of hIFIT1 and hIFIT3 on inhibition of the PIV5, MV, and NDV MG systems. The MG system plasmids were transfected into 293 IFIT1^{-/-} cells as before, together with the indicated IFITs expressed from pEF. 125ng of each pEF-IFIT plasmid was transfected per well of a 12-well plate for a total of 250ng pEF per well. Helper plasmid amounts were as follows: 8ng MG plasmid, 150ng pCAGGSV5-T7, 30ng pCAGGS-NP, 30ng pCAGGS-P, and 150ng pCAGGS-L. Error bars are SEM and data are an average of at least three independent replicates.

The data presented here provide strong circumstantial evidence for the cap structure of PIV5 mRNA being cap0. Assuming that the MV MG produces cap1 mRNA, there is a clear distinction between the effect of IFIT1 (particularly mIFIT1) on cap0 and cap1 mRNA with the putative cap0 structure of both PIV5 and NDV being strongly inhibited while the translation of an insensitive MG (MV) is actually increased by an unknown mechanism. The results seen with the MV MG also validate the inhibitory effect of IFIT1 upon the PIV5 MG because they show that co-expression of IFIT1 does not negatively affect MG helper gene expression (all the MG helper plasmids are the same pCAGGS vector).

3.3 PIV5 mRNA cap structure and L protein methyltransferase domain mutagenesis

The sensitivity of PIV5 to hIFIT1 and in particular mIFIT1 strongly suggest that its transcripts are incompletely methylated at the ribose of the first transcribed nucleotide because mIFIT1 is a well-characterised cap0-binding protein with minimal affinity for cap1. Interestingly, PIV5 L contains a putative MTase domain which closely aligns to other paramyxovirus L proteins and maintains the K-D-K-E catalytic tetrad required for MTase activity¹⁹⁴ (figure 3.22). This suggests that the PIV5 L protein should be able to generate cap1 structures.

The previous study by the Randall and Goodbourn groups of the sensitivity of PIV5 to IFIT1 attempted to determine the cap methylation status of PIV5 mRNA by using an in vitro cap methylation assay¹⁴⁹. Briefly, this involved isolation of total mRNA from infected cells and measurement of ³H-labelled S-adenosylmethionine (SAM) incorporation during a methylation reaction with the commercially-available Vaccinia virus cap1-methyltransferase, VP39^{255,256}. No incorporation of radiolabelled SAM was detected, so it was concluded that PIV5 transcripts are already 2'-O-methylated. However, the sensitivity of this assay is reduced by the majority of the isolated mRNA being of cellular origin and thus cap1. The insensitivity of the assay is exacerbated by the two most abundant viral transcripts (NP and P/V⁵⁰) being highly structured at the 5' end (see next chapter for evidence of this) which could make them poor substrates for VP39, as warned in the manufacturer's protocol. The level of unstructured transcripts (M and F) in the sample could be below the threshold of detection of incorporation of the radiolabel, making this experiment inconclusive. Furthermore, the positive control RNA used in this experiment (in vitro transcribed and N7-methyl-capped RNA encoding the NP of mouse hepatitis coronavirus) –

assuming it has the MHV 5'UTR – would have an unstructured 5' with a predicted 5-base overhang¹⁸⁰, making it a much better substrate for VP39 than PIV5 NP and P/V mRNAs.

In a follow-up study, some of these limitations were addressed and conflicting results were obtained by using a different approach¹⁵⁰. mRNA was isolated from PIV5-infected cells and treated with human cap methyltransferase 1 (CMTR1) before being in vitro translated in the presence or absence of recombinant hIFIT1. Inhibition of translation of NP mRNA was completely abolished by pre-treatment of the RNA with CMTR1. Interestingly, CMTR1 was unable to rescue translation of M mRNA in a similar manner. Despite this, the result seen with NP led us to conclude that PIV5 mRNA cannot be completely cap1-methylated because if it were, treatment with CMTR1 would have no protective effect on translation inhibition by IFIT1. This implies that either CMTR1 and VP39 differ in their requirement for a 5' overhang or that the approach taken in the previous study was simply less sensitive.

To address these ambiguities and the apparent paradox of PIV5 L having an MTase domain which fails to generate cap1, the cap structure of mRNAs in PIV5-infected cells was determined by mass spectrometry and the MTase domain of L investigated by mutagenesis.

3.3.1 CAP-MAP analysis of cap structures in PIV5-infected cells

The cap structure of all human mRNA is either cap1 or cap2, in a roughly equal ratio^{187,257}. This means that all cellular transcripts will have at the first position one of the following: Am, m6Am, Gm, Um, or Cm, with minimal or undetectable amounts of non-2'-O-methylated nucleotides²⁵⁸. Detection of cap0 structures in cells infected by a virus can therefore be attributed to the viral mRNA since levels of cap0 in uninfected cells should be negligible.

CAP-MAP (cap analysis protocol with minimal analyte processing) is a recently described method which uses liquid chromatography-mass spectrometry (LC-MS) to determine the relative abundances of cap structures of cellular mRNA²⁵⁹. Briefly, mRNA is selected from total cellular RNA and digested with the non-specific nuclease P1 which leads to release of both 5'monophosphate nucleotides and cap dinucleotides (i.e. m7GpppN), which are then resolved by LC and identified by MS. The methylation status of the first transcribed nucleotide (N) can be discriminated and thus the cap structure (cap0 or cap1) of mRNA in cells determined. Due to the use of commercially available cap dinucleotides as controls (e.g. m7GpppAm and m7GpppGm), only transcripts beginning with an A or a G are detected using this method.

CAP-MAP was employed to determine the relative abundance of cap0 structures in uninfected cells versus those infected with PIV5. This experiment was carried out by Elizabeth Wignall-Fleming (University of Glasgow; infection and RNA isolation) and Radoslaw Lukoszek (University of Dundee; LC-MS). mRNA was isolated from A549 cells either mock-infected, IFN-treated, or infected with PIV5(P/F157) at an moi of 10 at 24 hours post-infection, and cap dinucleotides quantified as described above with results shown as fmol of each dinucleotide per μg of mRNA (figure 3.21A). In both uninfected cells and IFN-treated uninfected cells, cap0 structures were barely detectable with m7GpppG and m7GpppA combined at 0.33 fmol/ μg and 0.53 fmol/ μg , just 0.10% and 0.17% of the total, respectively. In contrast, in cells infected with PIV5 the level of m7GpppA, but not m7GpppG, was dramatically increased to 60.95 fmol/ μg , resulting in the percentage of transcripts bearing cap0 increasing to 20.5% (figure 3.21B). All PIV5 mRNAs begin with an A so this structure is clearly of viral origin and not a change in cellular MTase activity as this would also result in m7GpppG, which was not seen. Furthermore, the proportion of cap0 transcripts (20.05%) following infection is approximately the proportion of viral to host sequences during PIV5 infection⁵⁰. Together, these data are strong evidence for PIV5 generating cap0, and not cap1 structures, on most or all of its transcripts.

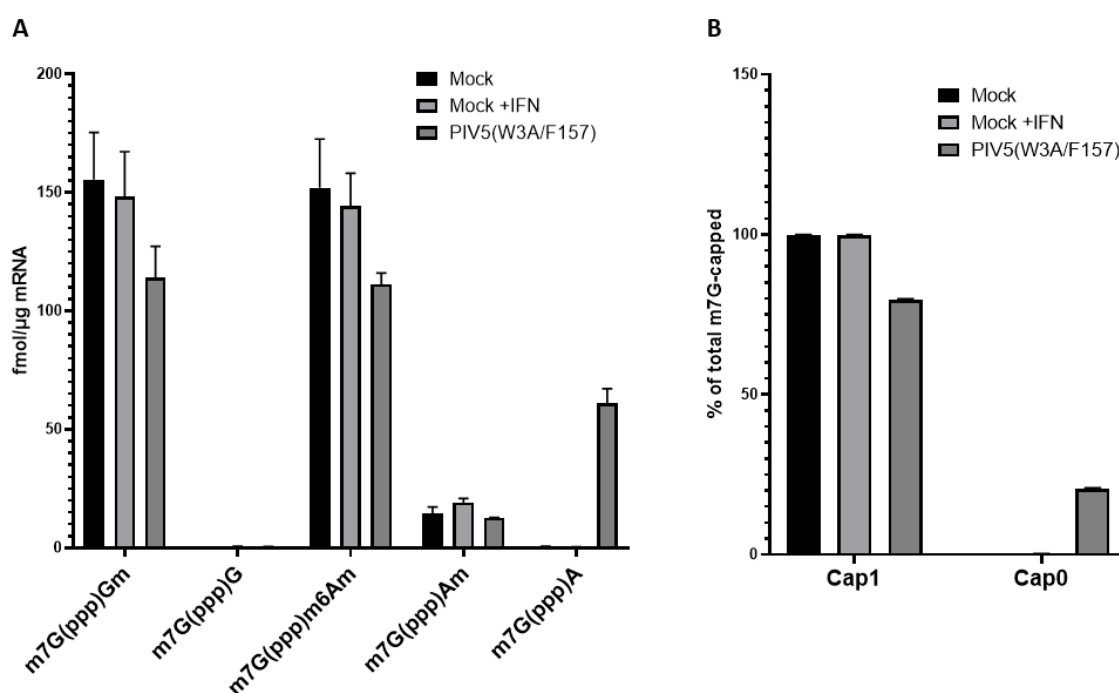


Figure 3.21 - CAP-MAP analysis of mRNA cap structure by LC-MS in infected cells. **A)** A549 cells were mock-infected, IFN-treated, or infected with PIV5(W3A/F157) at an moi of 10, and RNA isolated at 24hpi (Elizabeth Wignall-Fleming, University of Glasgow). Following selection of mRNA and digestion by nuclease P1, cap dinucleotides were resolved by LC and identified by MS (Radoslaw Lukoszek, University of Dundee). The amount of each cap dinucleotide per total mRNA is shown (fmol/ μg mRNA). **B)** The same data as in A with the cap1 (m7GpppGm, m7Gpppm6Am, m7GpppAm) and cap0 (m7GpppG, m7GpppA) structures combined and the percentage of cap1 or cap0 for each condition shown. For A and B, error bars are SEM and data are an average of two independent replicates.

A weakness of the CAP-MAP approach as employed above is that it is not specific for viral mRNA. Results are given as a combined total of cellular and viral mRNA, so while it is clear that the detected m7GpppA dinucleotide is of viral origin, it is not possible to determine what percentage of viral mRNA is cap0 or cap1. This percentage is likely to be very high but could be anywhere in a wide range (e.g. 50-100%) based on the obtained results. The ratio of cap0:cap1 could also vary between viral transcripts, which will be discussed at length in the next chapter. To address this issue, it was planned to repeat the experiment using viral mRNA selected by sequence-specific hybridisation probes, which would give both a percentage of cap0/1 for total viral transcripts and potentially also for each individual viral mRNA. However, this was not undertaken due to disruption caused by the coronavirus pandemic.

3.3.2 Alignment of *Mononegavirales* L MTase domains

Having established that PIV5 generates cap0 structures on most, if not all, of its transcripts, the question arises of how PIV5 transcription and cap methylation differs from that of non-*Rubulavirus* paramyxoviruses. *Mononegavirales* L proteins are extremely large, multifunctional proteins responsible for several enzymatic activities: RNA-dependent RNA polymerase, mRNA cap guanylyltransferase, mRNA cap guanine-N7-methyltransferase, and mRNA cap 2'-O-methyltransferase. The protein can be split into six separate domains responsible for different functions^{41,42}, with domain VI being responsible for both G-N7- and 2'-O-methylation of the mRNA cap²⁶⁰⁻²⁶². 2'-O-methyltransferases contain a K-D-K-E catalytic tetrad which is necessary for MTase activity^{188,263}, and a glycine-rich binding site (GxGxG) for the methyl donor S-Adenosyl methionine (SAM)^{264,265}. The boundaries of domain VI are not well-defined, but in PIV5 L domain VI can be considered to be in the region of amino acids 1731-2060³⁶.

An alignment of domain VI of paramyxovirus L proteins was made to identify any differences which may account for the defective 2'-O-methyltransferase activity of PIV5 L (figure 3.22). Residues widely conserved across all viruses are highlighted in yellow, with the K-D-K-E catalytic tetrad and the GxGxG SAM-binding motif indicated in red at the bottom of the alignment. Residues conserved in *Rubulavirus*, but not non-*Rubulavirus* L proteins, are highlighted in green; those conserved across non-rubulaviruses, but not in rubulaviruses, are highlighted in light blue. Although all of the aligned L proteins have an intact K-D-K-E catalytic tetrad and SAM-binding motif, at a cursory glance it appears that *Rubulavirus* L proteins have significantly diverged from non-*Rubulavirus* L proteins, with differences particularly prominent around the SAM-binding

motif. Most notable is the difference at the first position of the SAM-binding motif itself, with rubulaviruses having AxGxG rather than GxGxG.

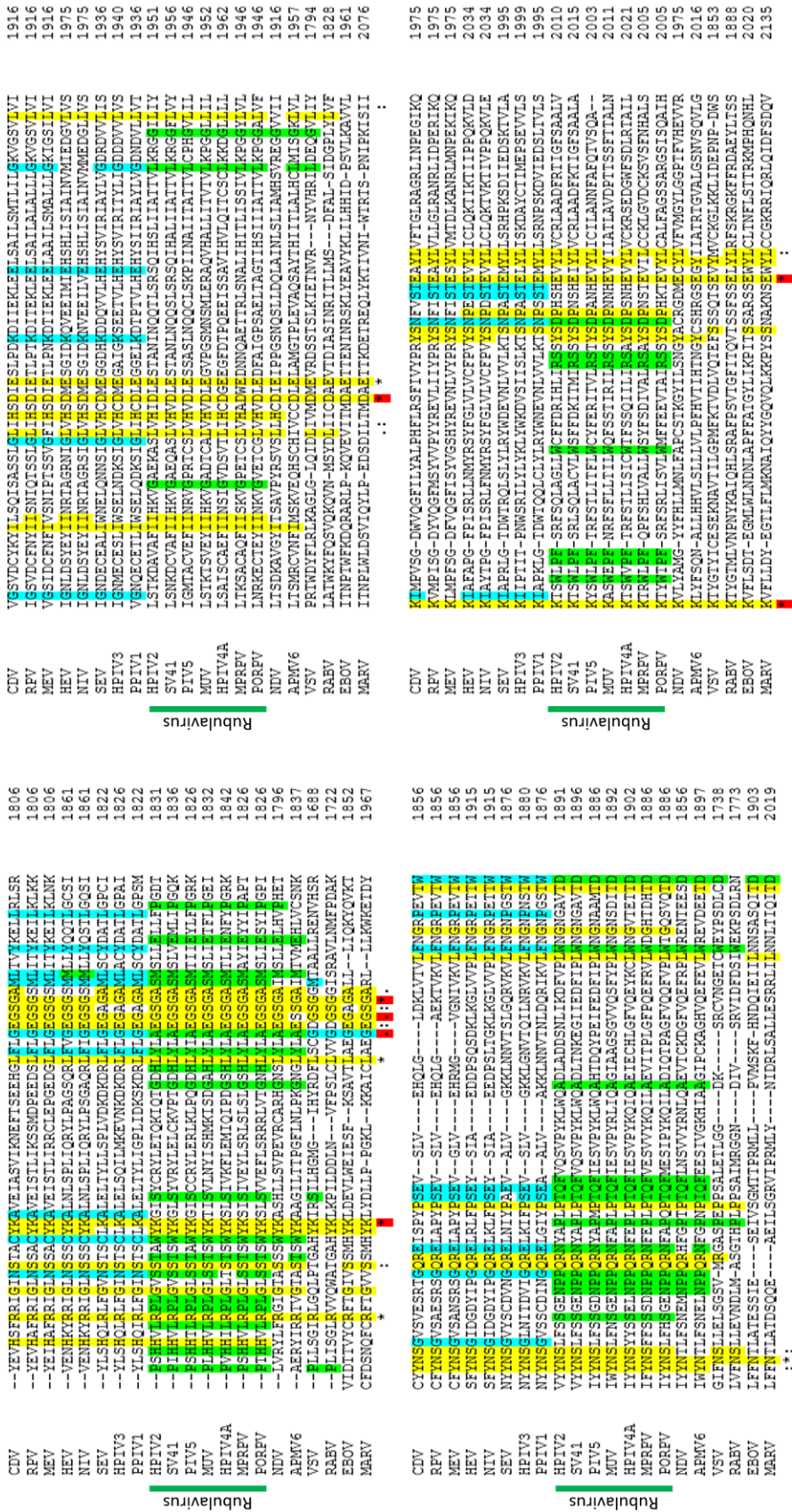


Figure 3.22 - Protein sequence alignment of the methyltransferase domain (domain VI, amino acids 1778-1994 in PIV5) of Mononegavirales. All are Paramyxoviridae except for VSV and RABV (Rhabdoviridae), and EBOV and MARV

(*Filoviridae*). Extensively conserved residues are highlighted in yellow; residues conserved within the *Rubulavirus* genus are highlighted in green; residues conserved amongst non-*Rubulavirus* paramyxoviruses are highlighted in blue. The K-D-K-E catalytic tetrad and the GxGxG SAM-binding motif are highlighted in red below the sequence. Full names of the viruses shown can be found in the abbreviations section. Alignment made with Clustal Omega. GenBank reference number for each L protein can be found in the methods (section 2.5).

The most thorough characterisation of a paramyxovirus L MTase domain to date has been for SeV. Mutational analysis of numerous conserved residues was performed and the relative importance of each investigated by virus reverse genetics, growth curves, and quantification of MTase activity^{266,267}. The methodology employed in these studies was unable to distinguish between guanine-N7 and 2'-O-adenosine methylation. One report of an in vitro assay of SeV L protein cap methylation detected guanine-N7 MTase activity but not 2'-O-adenosine activity²⁶⁸, and in the SeV studies there is some weak evidence that SeV mRNA is methylated only at the guanine-N7 position²⁶⁷, but this conflicts with previous reports of SeV generating cap1 structure²⁶⁹ and more recent evidence of SeV being insensitive to IFIT1¹⁵⁰. Nevertheless, Murphy *et al.* identified multiple residues important for MTase activity, including those of the K-D-K-E catalytic tetrad and at positions close to the SAM-binding motif. However, the GxGxG sequence of the SAM-binding motif itself was tolerant of mutations and substituting alanines for the glycines had no negative impact on MTase activity or viral growth. It is therefore unclear whether the AxGxG sequence present in the rubulavirus MTase domain (see figure 3.22) would have any effect on MTase activity, or whether it could explain the lack of 2'-O-methylation of PIV5 mRNAs. Interestingly, Murphy *et al.* also found that VSV mutants defective in cap methylation were attenuated in Hep-2 cells but were able to grow in BSRT7 and Vero cells, both of which are defective in IFN production^{270–273}.

A more recent study of the -ssRNA human metapneumovirus (hMPV, family *Pneumoviridae*) used an in vitro assay in which methylation of short, partially methylated RNA substrates by the hMPV L protein was measured²⁷⁴. This approach was able to distinguish between G-N7- and 2'-O-MTase activity and found that while most mutations in domain VI had a similar effect on both, mutation of the second lysine of the K-D-K-E catalytic tetrad to alanine (K1817A in hMPV L) resulted in loss of only 2'-O-methylation.

The inhibition of the PIV5 MG system by hIFIT1 and mIFIT1 – and the complete lack of inhibition of the MV MG system – potentially provides a model to investigate L protein 2'-O-MTase activity. Any increase in cap1 structure generation by the PIV5 MG will desensitise it to IFIT1, while loss of cap1 structure in the MV MG will sensitise it to IFIT1. Therefore, a mutagenesis analysis was undertaken for both PIV5 L and MV L based on the alignments in figure 3.22 and informed by the previous work on SeV L and hMPV L. The residues selected for mutation are shown in figure 3.23 along with the rationale for each, and are discussed in more detail in the following sections.

PIV5		MV	
Mutation	Rationale	Mutation	Rationale
Wt	Wt L of PIV5 strain W3A	Wt	Wt L of MV strain Edmonston
P5MV	Chimaeric L with MV MTase domain	K1766A	K-D-K-E catalytic tetrad
K1786A	K-D-K-E catalytic tetrad	D1881A	
D1911A		K1917A	
K1947A		E1954A	
E1985A		F1786Y	SAM-binding motif
Y1806F	SAM-binding motif	G1788A	
I1807L		M1793S/L1794M	
A1808G		C1764W	Other differences versus rubulaviruses
S1814M/M1815L	Other differences versus non-rubulaviruses	G1820P/E1823N	
W1784C		W1856D	
P1840G/N1843E		N1949D	
D1886W			
D1979N			
T1773I	SNPs indentified in Rima <i>et al.</i> 2014		
D1979G			

Figure 3.23 - Mutations made in the L proteins of PIV5 and MV. Mutations were made to the K-D-K-E motif, the SAM-binding motif and surrounding residues, several conserved differences between rubulaviruses and non-rubulaviruses, as shown in figure 3.22, and for PIV5 L two SNPs identified in a previous study. P5MV consists of the N-terminal 1777 amino acids of PIV5 L fused to the MTase (domain VI) and CTD of MV L.

3.3.3 Mutagenesis of the PIV5 L MTase domain

The following amino acids of PIV5 L were chosen for analysis: those of the K-D-K-E motif (K1786, D1911, K1947, and E1985); the first position of the SAM-binding motif (A1808); several differences compared to non-rubulaviruses around the SAM-binding motif (Y1806, I1807, S1814/M1815), around the residues of the catalytic tetrad (W1874 and D1979), and at other locations (P1840/N1843 and D1886); and variants resulting from SNPs within the L gene (T1773 and D1979) previously detected during passage of PIV5 in cell culture⁶⁹. An alanine was substituted for K-D-K-E motif residues while for the other changes, either the equivalent found in non-rubulaviruses or the minor SNP variants were substituted. Additionally, a chimaeric L (P5MV) was made in which domain VI and the CTD of PIV5 L were replaced with that of MV L so that the first 1730 amino acids are from PIV5 L, and the last 463 amino acids are from MV L; if functional, a chimaeric L protein may be able to synthesise cap1 structure on PIV5 mRNAs. A summary of all these changes is shown in figure 3.23.

An initial test of the activity of each L protein mutant was made in the MG system with the high-activity P(CPI+). As shown in figure 3.24, most mutants retained some level of activity, though W1784C, P1840G/N1843E, and D1886W were completely non-functional, as was the P5MV chimaeric L. Interestingly, A1808G was active to only about 40% of Wt L, suggesting that the apparent loss of the GxGxG SAM-binding motif in rubulaviruses may promote polymerase activity. Also of note is that the naturally-occurring D1979G variant has an activity of only about

30% of Wt L. This SNP warrants further investigation in light of the finding that mutations in P which have a negative effect on viral RNA synthesis are potentially selected for during infection to promote persistence²²⁴; a similar mechanism has not been described in the L protein but could have a comparable effect on viral phenotype. An attempt was made to detect all the L mutants by western blot (all L proteins here are N-terminally V5-tagged) but no protein was detected; the L protein is extremely large (256 kDa), making detection by western blot difficult.

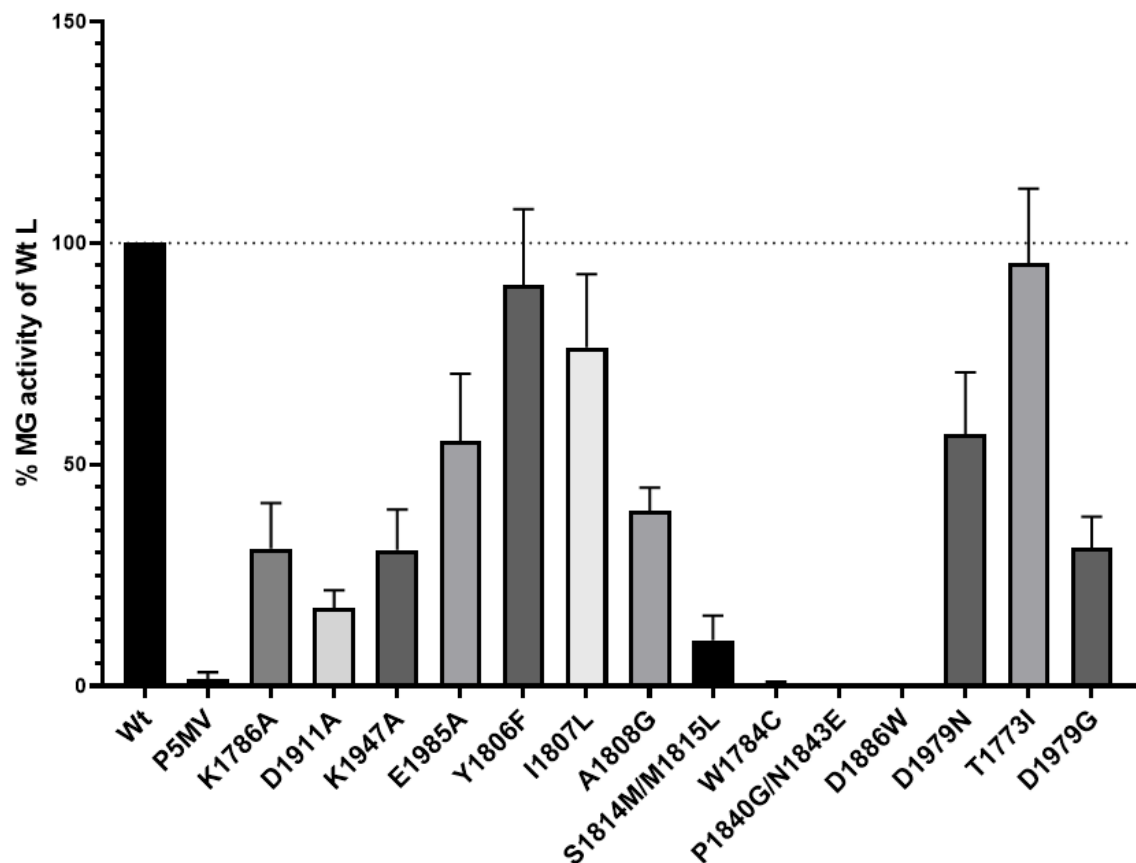


Figure 3.24 - Activity of PIV5 L mutants in the MG assay in 293 IFIT1^{-/-} cells. V5-tagged L mutants were tested in the MG assay as previously described. Results are expressed as a percentage of the activity of Wt L. Error bars are SEM and data are an average of at least three independent replicates.

Next, the sensitivity of the active PIV5 L mutants to hIFIT1 and mIFIT1 was tested (figure 3.25). No difference in sensitivity was seen in any L mutant, with hIFIT1 inhibiting activity to about 50% and mIFIT1 inhibiting to >10%, as seen in the previous sections. The failure of the Y1806F, I1807L, A1808G, and D1979N changes to affect sensitivity to IFIT1 suggests that it is unlikely that any single amino acid change can increase the 2'-O-MTase activity of PIV5 L, though this is obviously not an exhaustive analysis of conserved residues that differ between PIV5 and non-rubulaviruses. More informative is the lack of effect on IFIT sensitivity of mutation in the K-D-K-E catalytic tetrad. If PIV5 L generated cap1 mRNA to any extent, loss of any of the K-D-K-E residues would

be expected to increase sensitivity to hIFIT1. Instead no difference was seen, suggesting that the MG Fluc transcripts generated by Wt L are all cap0.

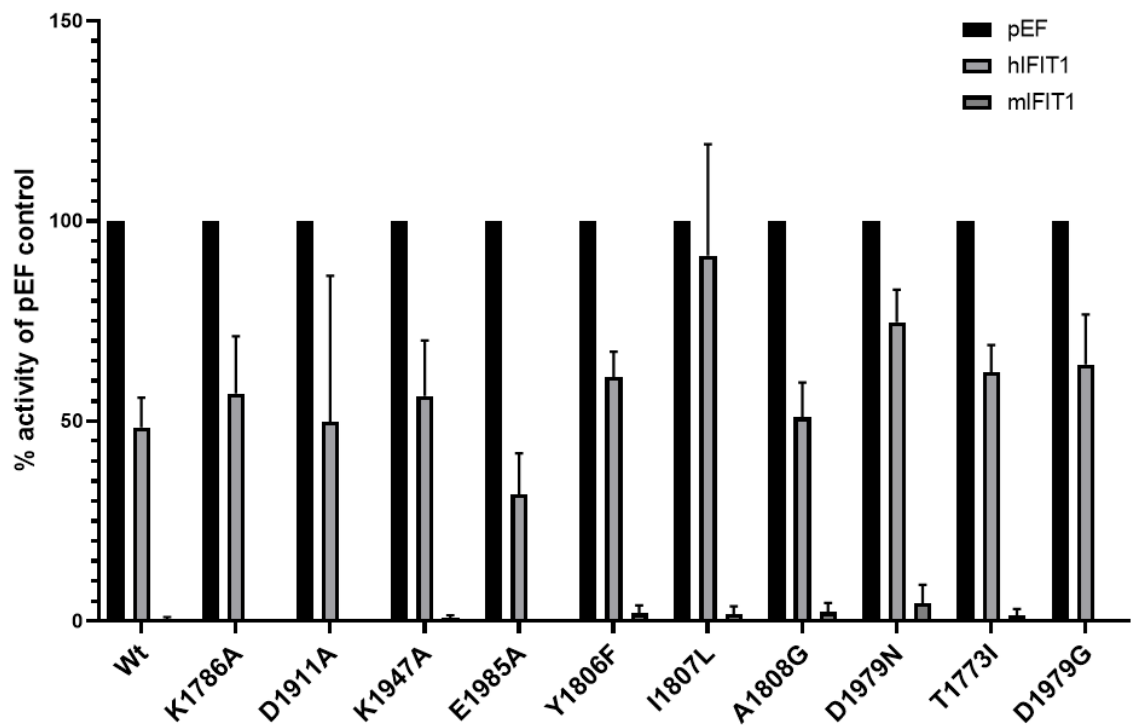


Figure 3.25 - Sensitivity of PIV5 L mutants to IFIT1 in the MG assay in 293 IFIT1^{-/-} cells. Active L mutants were tested in the MG system with co-expressed hIFIT1 and mIFIT1 (125ng of pEF per well of a 12-well plate). Results are expressed as a percentage of the activity of the empty pEF vector control. Error bars are SEM and data are an average of at least three independent replicates.

3.3.4 Mutagenesis of the MV L MTase domain

No single amino acid change was found to restore PIV5 L 2'-O-MTase activity (as inferred by sensitivity to IFIT1). Therefore, the equivalent changes were made in MV L and tested using the MV MG system which is insensitive to IFIT1. This approach is perhaps more promising because single amino acid changes are more likely to disrupt enzymatic activity than restore it. The following amino acids of MV L were chosen for analysis: those of the K-D-K-E motif (K1766, D1981, K1917, and E1954); the first position of the SAM-binding motif (G1788); and several differences compared to rubulaviruses around the SAM-binding motif (F1786 and M1793/L1794), the residues of the catalytic tetrad (W1764 and N1949), and at other locations (G1820/E1823 and W1856).

Unfortunately, all of the changes made to MV L severely affected its activity in the MV MG system (figure 3.26A). Fluc signal was not detectable above background levels for any changes

except K1766A, G1788A and G1820P/E1823N, which were even then only active to 2%, 4%, and 23% of Wt L, respectively, and which showed no difference in sensitivity to IFIT1 (figure 3.26B). The deleterious effect of domain VI mutations on MV L is in contrast to PIV5 L for which many of the equivalent changes maintained activity. It is not clear why this is the case, but one could speculate that the MTase domain of MV L is more important for viral translation than the possibly non-functional MTase domain of PIV5 L. However, it is doubtful that loss of 2'-O-methylation alone would have such a drastic effect on activity because cap1/2 methylations are not required for translation of cellular transcripts in most conditions^{186,189} and the experiment was carried out in 293 IFIT1^{-/-} cells which lack the only ISG known to restrict cap0 mRNA. It is possible that cap0 MV MG transcripts are degraded by host mRNA quality control mechanisms, for example by DXO²⁷⁵, but this is unlikely to affect mRNAs transcribed in the cytoplasm and would also be expected to affect the PIV5 MG, as would RIG-I-dependent induction of IFN by cap0 mRNA^{276,277}. As for the PIV5 L mutants, the MV L mutants were unable to be detected by western blot due to the large size of the MV L protein (248 kDa).

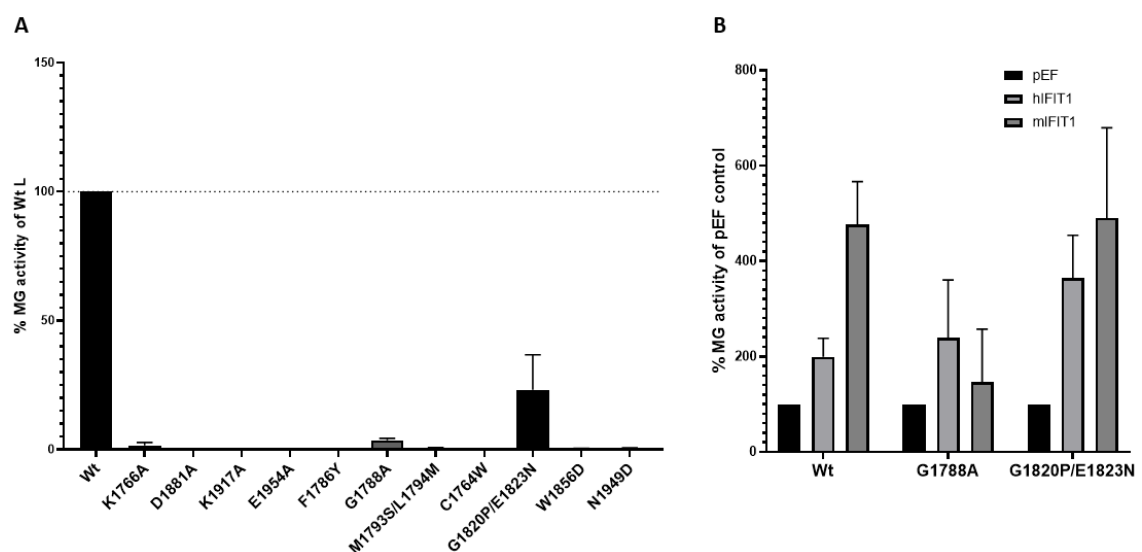


Figure 3.26 - Activity and IFIT sensitivity of MV L mutants in the MG assay. **A)** V5-tagged L mutants were tested in the MG assay as previously described. Results are expressed as a percentage of the activity of Wt L. **B)** Active L mutants were tested in the MG system with co-expressed hIFIT1 and mIFIT1. Results are expressed as a percentage of the activity of the empty pEF vector control. For both graphs, error bars are SEM and data are an average of at least two independent replicates.

Therefore, the mutations in MV L are likely either disrupting protein folding or affecting another function of the enzyme such as cap guanine-N7-methylation. G-N7 methylation is not required for translation in in vitro translation systems¹⁵⁰, but in the cellular environment mRNA lacking G-N7 methylation is unstable¹⁸³ and poorly translated due to being outcompeted for binding to

translation factors¹⁸⁴. Although not investigated further, a lack of translation due to incomplete G-N7 methylation could be assayed using a MG in which a second reporter gene is expressed from an IRES on the same transcript as the first reporter gene; in this way, the first reporter would provide a readout of cap-dependent translation, while the second would serve as cap-independent translation control.

If mutations in the MTase domain of MV L are affecting G-N7 methylation, the question arises of why changes to the PIV5 L MTase domain do not have the same effect, in particular the mutations of the K-D-K-E catalytic tetrad. One explanation could be that for MV L, 2'-O-methylation precedes and is required for G-N7 methylation, and that loss of any of the K-D-K-E residues indirectly affects the latter by inhibiting the former. The flavivirus NS5 protein sequentially methylates mRNA at the G-N7 and 2'-O positions¹⁹⁹ and these processes differ in their requirement for an intact K-D-K-E motif²⁷⁸. For example, mutation of the glutamic acid of the K-D-K-E tetrad in the NS5 of West Nile virus (WNV) results in a virus which produces cap0 mRNA and is attenuated due to IFIT1, but maintains G-N7 MTase activity and is therefore not attenuated in the absence of IFIT1^{130,279}. Similar results have also been reported with MTase mutants of coronaviruses^{178,280} and poxviruses^{161,281}. In contrast, K-D-K-E mutations in SeV L cause a complete loss of both G-N7 and 2'-O methylation^{266,267}, as do the equivalent mutations in VSV L^{261,262}. A potential explanation for this difference between positive and negative sense RNA viruses is that *Mononegavirales* L proteins have been proposed to sequentially methylate mRNA at the 2'-O and then the G-N7 positions in such a way that the former is required for the latter. This has been shown most thoroughly for VSV L^{196–198}, but has also been suggested for hMPV L²⁷⁴, EBOV L²⁸², and RSV L²⁸³.

For paramyxoviruses, such as PIV5 and NDV, which produce cap0 mRNA, this sequential mechanism of 2'-O and G-N7 methylation would be disrupted. Presumably, the MTase domain of these viruses has evolved to utilise a different mechanism to permit G-N7 methylation in the absence of prior 2'-O methylation, which could explain the apparent significant divergence of the MTase domains of rubulaviruses and avulaviruses from other *Mononegavirales*, as shown in figure 3.22. Such a mechanism of methylation could also explain why mutations in the K-D-K-E motif of PIV5 L do not abolish MG activity as they do in MV L presumably due to loss of G-N7 methylation. In this scenario, the variant SAM-binding motif (AxGxG) in rubulaviruses and avulaviruses could represent part of this adaptation, possibly due to an altered affinity for SAM²⁶⁷ and potentially demonstrated by the reduced activity of A1808G in the PIV5 MG (figure 3.24).

Phylogenetic analyses of paramyxoviruses show that viruses of the *Rubulavirus* and *Avulavirus* genera are more closely related to each other than the other paramyxovirus genera^{5,284,285}. Given

the lack of IFIT1 in birds, the natural hosts of avulaviruses¹⁶⁰, there may have been insufficient selective pressure on these viruses to maintain viral 2'-O-MTase activity. A hypothetical shared evolutionary history between avulaviruses and rubulaviruses could explain why both genera fail to generate cap1 structures. The apparent lack of selective pressure on avulaviruses to maintain 2'-O-MTase activity is made clear by the alignment shown in figure 3.27. Several members of this genus have lost one or more of the highly conserved residues of the K-D-K-E tetrad. This will be discussed further in chapter 5.

NDV	SSSWYKASH--LLSVPEVRCA	1769	NDV	LLHCDIEIPPGSN	1889
APMV2	STSWYKYAV--VGASVEKVQP	1809	APMV2	RVVCEVEFDRGMP	1929
APMV3	HSVWYRNRQYKLILSQLSHDI	1762	APMV3	RIICDIELARDTS	1876
APMV4	SSAWYRSAA--LLASGALNGL	1780	APMV4	LIHVDIEFSPDSN	1894
APMV5	SGSWYKISG--LLAKGLCKGL	1832	APMV5	ISVCDLELSFGTP	1952
APMV6	STSWYKAAG--ILTTPGFLNL	1810	APMV6	IVCCDLELAMGTP	1930
APMV7	STAWYKASS--LIASDILKGG	1797	APMV7	LIHADLDLPIETP	1917
APMV8	STSWYKYAI--AYAVSEKRSA	1809	APMV8	RVNCDVEFDRGMP	1929
APMV9	SSSWYKAAN--LLSLPEVRQA	1772	APMV9	LLHCDIETTLEPS	1892
APMV10	STSWYKYAV--LYASERKTAK	1807	APMV10	RVNCDVEFDEGMP	1927
APMV11	STSWYKYAG--LYSSEFFRGM	1815	APMV11	LVVVEAEFERGLS	1935
APMV12	SSSWYKIGQ--LFSIPEVRQS	1769	APMV12	FLMVD AELPSDSP	1889
APMV13	SSSWYKVGQ--IFS IPEVRQT	1769	APMV13	FIMVDAEIPKDSA	1889
APMV15	STSWYKFAV--WYATRMQRQSV	1809	APMV15	RIVCDAEFD SGMP	1929
	**:			:	
NDV	VV I I K V L Y A M G	1923	NDV	R G D M E C Y L V F V	1960
APMV2	R L I I K V Y R H S E	1963	APMV2	T N K E E Y I I I A A	2000
APMV3	I C I C R C H L L N A	1910	APMV3	V N D P S C V V G T T	1947
APMV4	R F A V R V F L S D H	1928	APMV4	K D D G A G Y I C A K	1947
APMV5	P G V L K L Y F T H V	1986	APMV5	Q E G R E C Y I V F R	2023
APMV6	K L V L K L Y F S Q N	1964	APMV6	H R G S E G Y I I A T	2001
APMV7	K L V M K L Y A V D E	1951	APMV7	D K S K E C Y L I G T	1988
APMV8	I L W V K V Y R T S E	1963	APMV8	A K D E E Y I L M S D	2000
APMV9	V F I I K V L Y T Q S	1926	APMV9	R G D F E C Y L V A T	1963
APMV10	H L W V K V Y R S S E	1961	APMV10	P N H E E F V L A C K	1998
APMV11	R F V V K V N A I D T	1969	APMV11	L E Q K E V L V V G S	2006
APMV12	V M I I K A L F T R T	1923	APMV12	R G D G E C Y I I G W	1960
APMV13	V L V I K T L Y S Q C	1923	APMV13	R G D Y E C F L I A I	1960
APMV15	L I L L K L H C T S E	1963	APMV15	L N N Q E I L V S A T	2000
	:			:	

Figure 3.27 - Protein sequence alignment of the K-D-K-E catalytic tetrad residues of Avulavirus L proteins, shown in yellow when present. Full names of the viruses shown can be found in the abbreviations section. Alignment was made with Clustal Omega. GenBank reference number for each L protein can be found in the methods (section 2.5).

The inability to make a mutant MV L protein specifically defective in 2'-O-methylation means that it is not possible to perform the informative experiments that have been conducted with flaviviruses and coronaviruses. It would be ideal if it were possible to make changes to PIV5 L that restore 2'-O-methyltransferase activity but this is unlikely to be achieved by single amino acid changes and chimaeric proteins with switched MTase domains are unlikely to be functional due to compatibility issues both within the protein³⁶ and with other viral proteins²⁸⁶, as shown by the inactivity of the P5MV L protein (figure 3.24). An in-depth characterisation of the

structure and enzymatic activity of the PIV5 MTase domain will be needed to determine how and why it differs from that of non-rubulaviruses.

3.4 The sensitivity of PIV5 to IFIT1 during infection

Thus far, the sensitivity of IFIT proteins has been investigated using the MG system. While this has been validated as an appropriate model to study sensitivity by using controls and comparing to other viral MGs, it does not completely recapitulate the environment encountered by the virus during an IFN-induced antiviral response. Important differences include the amount of IFIT1 induced by IFN compared to that expressed by transfection; the additional induction of other ISGs which may act cooperatively with IFIT1; and the presence of viral proteins that are not part of the MG system and which may have roles in innate immune evasion. Therefore, in the following sections the importance of IFIT1 during PIV5 infection is investigated through use of IFIT1 knockout cells and a virus expressing the fluorescent protein mCherry.

3.4.1 Generation of IFIT1 knockout cells using CRISPR-Cas9

CRISPR-Cas9 was used to generate IFIT1 knockout clones of 293 and A549 human cells, and BALB/3T3 mouse cells. The 'double nickase' approach was used to minimise off-target effects²¹². The A549 cells used for this have previously been engineered to express GFP under control of the IFN- β promoter (A549/pr(IFN- β).GFP)¹²⁰ but this feature was not used during these experiments and the cells will simply be referred to as A549 or A549 IFIT1^{-/-}. The sgRNA target sequences for both hIFIT1 and mIFIT1 are shown in the figures below (figures 3.28-30), underlined in the unedited sequence.

To confirm knockout of hIFIT1 following selection (puromycin resistance) and clonal expansion, gDNA was isolated from 293 and A549 clones and the edit site amplified using primers binding either side of it which produce a short (170-250 bp product) in the unedited genome. Insertions or deletions at the edit site were then detected by analysing the PCR products on a high-percentage agarose gel. The PCR products were purified from the gel and sequenced to check whether the insertions/deletions have caused a frameshift in the IFIT1 CDS, as shown in the figures. Finally, loss of IFIT1 protein production was confirmed by treating cells with IFN and detecting IFIT1 by western blot at 18h post-treatment. In unedited control cells, IFIT1 was easily detectable but in both A549 IFIT1^{-/-} (figure 3.28B) and 293 IFIT1^{-/-} (figure 3.29B), no IFIT1 was

detected following IFN-treatment, confirming that knockout has been successful. BALB/3T3 mIFIT1^{-/-} cells were confirmed in the same way except that no western blot was performed. DF-1 chIFIT5^{-/-} cells were previously made and characterised by Craig Ross (St George's, University of London).

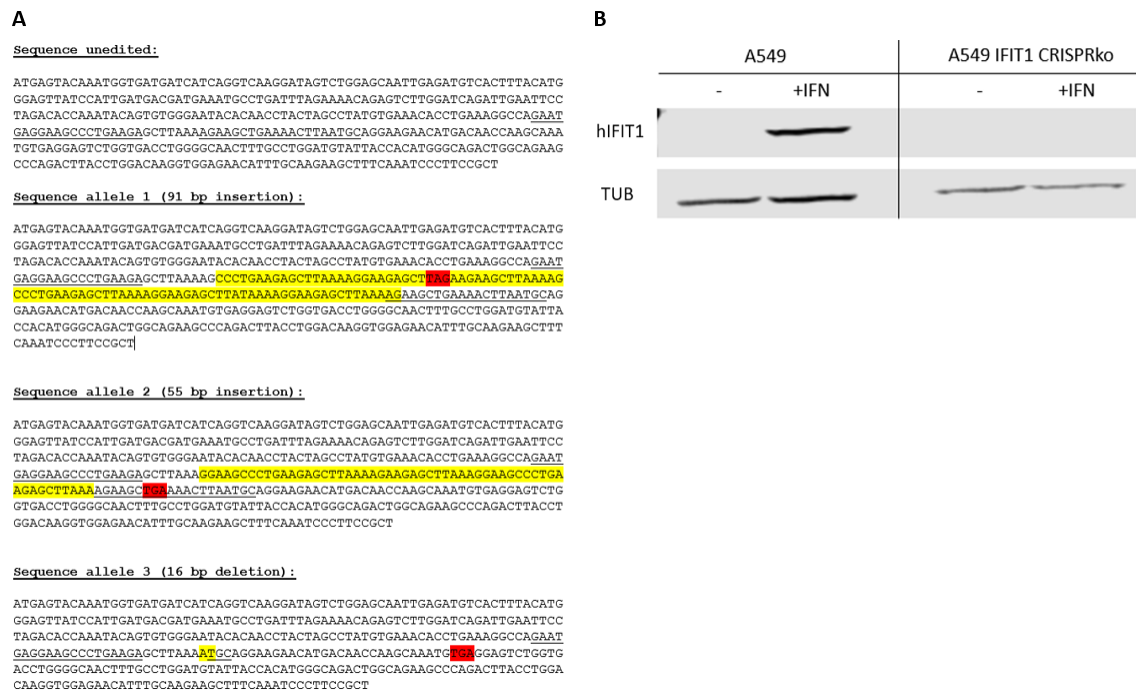


Figure 3.28 - CRISPR-Cas9-mediated knockout of hIFIT1 in A549 cells. A) The first 400bp of the CDS of IFIT1 are shown with the sgRNA target sequences underlined. For the three edited alleles, inserted sequences are shown in yellow. Deleted sequences are marked by two bases highlighted in yellow with the deleted region being between them. The first stop codon present in the shifted reading frame is shown in red. **B)** A549 and A549 IFIT1^{-/-} cells were treated with IFN and processed for western blotting at 18h post-treatment with rabbit anti-IFIT1 and mouse anti-tubulin.

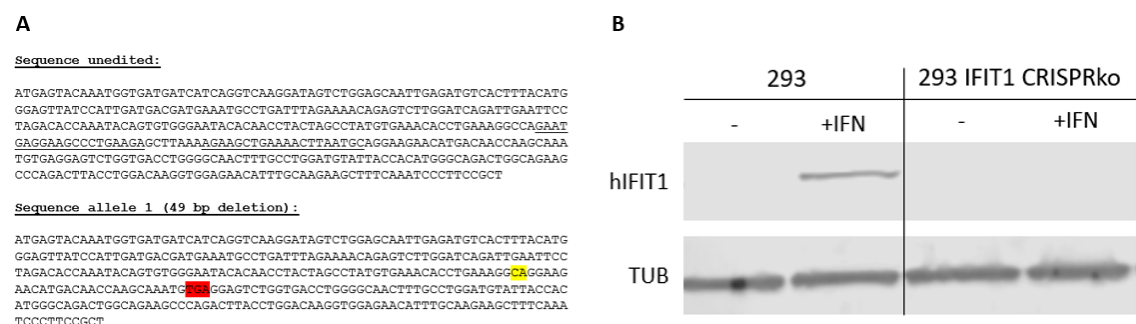


Figure 3.29 - CRISPR-Cas9-mediated knockout of hIFIT1 in 293 cells. A) The first 400bp of the CDS of IFIT1 are shown with the sgRNA target sequences underlined. The deleted sequence is marked by two bases highlighted in yellow with the deleted region being between them. The first stop codon present in the shifted reading frame is shown in red. **B)** 293 and 293 IFIT1^{-/-} cells were treated with IFN and processed for western blotting at 18h post-treatment with rabbit anti-IFIT1 and mouse anti-tubulin.

Sequence unedited:

ATGGGAGAGAATGCTGATGGTGACCAGGTCATGGAGAATCTGCTTCAGCTGAGATGTCACCTTCACATG
GAAGCTGCTATTGAAAATAATGACATACCTGATTGGAAAGTGAGAATCTCAGAGCAGGTCCAGTTCC
TTGACATCAAGAACCCATTGGGGATGCACAACCTCCTGGCCTACGTGAGGCACCTGAAAGGCCAGCAG
GACGAAGCCCTGCAGAGCTTGAAGAAGCTGAAGCCTTGATCCAGAGCGAGCAGCTGAGCAAGAGAAG
CCTGGCGACCTGGGGCAACTGTGCTGGCTGCATTACACAGGGGCAGCTTGGCAGAAGGCCAGATCT
ACCTGGACAAGGTGGAGAAGGTGTGAAGGAATTTCAAGTCCCTTCGCTACAGGCTGG

Sequence allele 1 (25 bp deletion):

ATGGGAGAGAATGCTGATGGTGACCAGGTCATGGAGAATCTGCTTCAGCTGAGATGTCACCTTCACATG
GAAGCTGCTATTGAAAATAATGACATACCTGATTGGAAAGTGAGAATCTCAGAGCAGGTCCAGTTCC
TTGACATCAAGAACCCATTGGGGATGCACAACCTCCTGGCCTACGTGAGGCACCTGAAAGGCCAGCAG
AAGCTGAAGCCTTGATCCAGAGCGAGCAGCTGAGCAAGAGAAGCCTGGCGACCTGGGGCAACTGTGCC
TGGCTGCATTACACAGGGGCAGCTTGGCAGAAGGCCAGATCTACCTGGACAAGGTGGAGAAGGTGTG
CAAGGAATTTCAAGTCCCTTCGCTACAGGCTGG

Sequence allele 2 (40 bp deletion):

ATGGGAGAGAATGCTGATGGTGACCAGGTCATGGAGAATCTGCTTCAGCTGAGATGTCACCTTCACATG
GAAGCTGCTATTGAAAATAATGACATACCTGATTGGAAAGTGAGAATCTCAGAGCAGGTCCAGTTCC
CTAGCCTGGCACCTGAAAGGCCAGCAGGACGAAGCCCTGCAGAGCTTGAAGAAGCTGAAGCCTTGA
TCCAGAGCGAGCAGCTGAGCAAGAGAAGCCTGGCGACCTGGGGCAACTGTGCTGGCTGCATTACAC
ATGGGGCAGCTTGGCAGAAGGCCAGATCTACCTGGACAAGGTGGAGAAGGTGTGAAGGAATTTCAAG
TCCCTTCGCTACAGGCTGG

Figure 3.30 - CRISPR-Cas9-mediated knockout of mIFIT1 in BALB/3T3 cells. The first 400bp of the CDS of IFIT1 are shown with the sgRNA target sequences underlined. The deleted sequences are marked by two bases highlighted in yellow with the deleted region being between them. The first stop codon present in the shifted reading frame is shown in red.

3.4.2 Infection of knockout cells with PIV5/F157/mCherry

IFIT1 has previously been shown to inhibit PIV5 translation and to be the main human ISG responsible for sensitivity of PIV5 to IFN, as determined by both increased viral protein level and plaque size in cells in which IFIT1 has been knocked down^{149,150}. To confirm these results with A549 IFIT1^{-/-} cells, a PIV5 virus expressing mCherry from an additional gene inserted between HN and L was used to provide a readout of viral translation. This virus is the W3A strain with a single mutation in P/V (S157F) which increases viral polymerase activity^{38,224} and is therefore named PIV5/F157/mCherry.

A549, BALB/3T3, and DF-1 cells were pre-treated with IFN and then infected with PIV5/F157/mCherry at an moi of 10 and imaged at 18hpi. This provides a simple means of assessing viral translation following pre-treatment with IFN. In agreement with the published data, pre-treatment of A549 cells with IFN resulted in a drastic reduction of mCherry intensity, while in hIFIT1 knockout cells mCherry intensity was nearly indistinguishable between treated and untreated cells (figure 3.31). This both emphasises the inhibitory effect of hIFIT1 on PIV5 mRNA translation and implies that other human ISGs have at most a minor inhibitory effect on PIV5 transcription and translation.

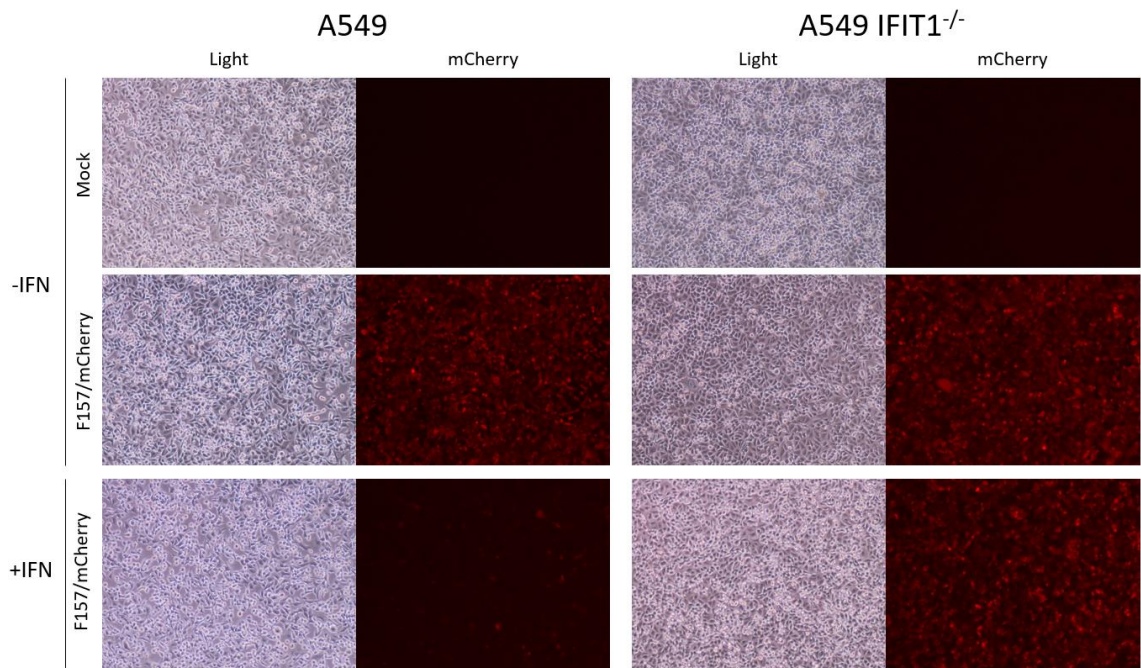


Figure 3.31 - Infection of A549 and A549 IFIT1^{-/-} cells with PIV5. Confluent cells were or were not pre-treated with IFN at 8h pre-infection, and then infected with PIV5/F157/mCherry at an moi of 10. At 18hpi, images were taken with a fluorescent microscope.

In both mouse and chicken cells, however, the role of IFIT1 and IFIT5 was not as clear (figures 3.32 and 3.33). It is important to note that PIV5 does not naturally infect either mice or chickens, but due to its use of sialic acid as a receptor for entry, it has a non-specific cell tropism; growth in these cells is attenuated but initial viral transcription and translation still occur. Pre-treatment of mouse BALB/3T3 cells with universal IFN resulted in a noticeable reduction in the number of mCherry-positive cells, though the intensity of mCherry in cells that were positive was comparable to untreated cells. Knockout of mIFIT1 did not rescue mCherry expression to the same extent seen with knockout of hIFIT1 in A549 cells, though there was an increase in the number of mCherry-positive BALB/3T3 IFIT1^{-/-} cells after IFN treatment relative to BALB/3T3. These cells were only faintly mCherry-positive, suggesting that mIFIT1 is only partially responsible for the IFN-sensitivity of PIV5 in mouse cells. A similar result was seen in chicken cells; PIV5 was highly sensitive to pre-treatment of DF-1 cells with chicken IFN and knockout of chIFIT5 provided only a minimal rescue of mCherry expression. These results imply that in contrast to human cells, mouse and chicken ISGs other than IFITs are able to restrict PIV5 transcription and translation.

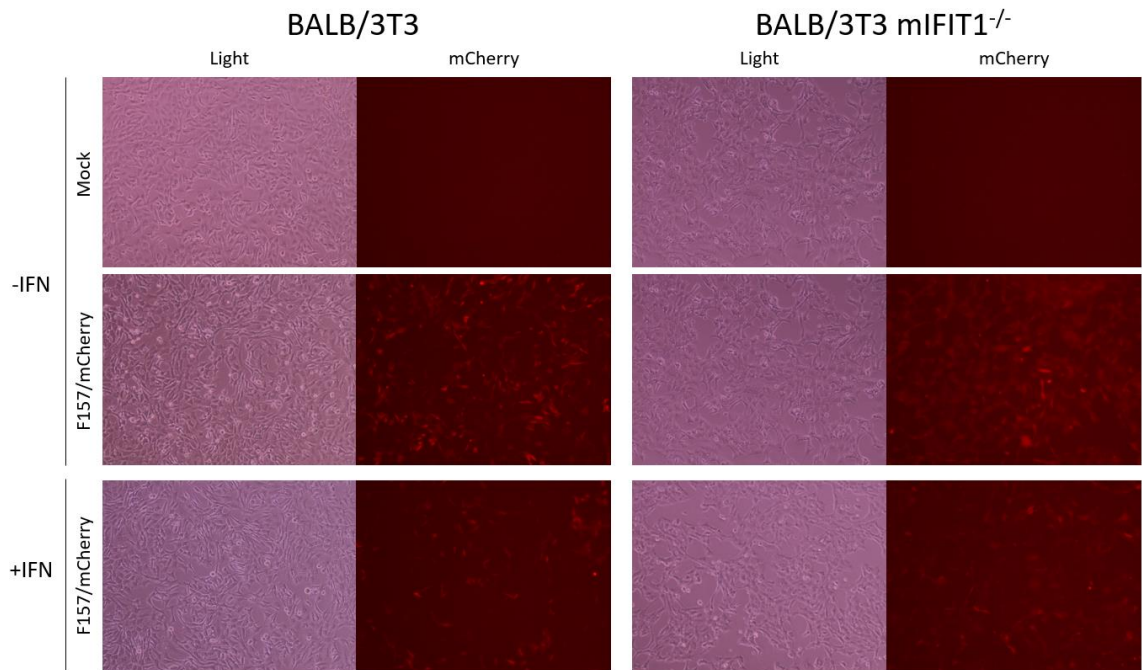


Figure 3.32 - Infection BALB/3T3 and BALB/3T3 mIFIT1^{-/-} cells with PIV5. Confluent cells were or were not pre-treated with universal IFN at 8h pre-infection, and then infected with PIV5/F157/mCherry at an moi of 10. At 18hpi, images were taken with a fluorescent microscope.

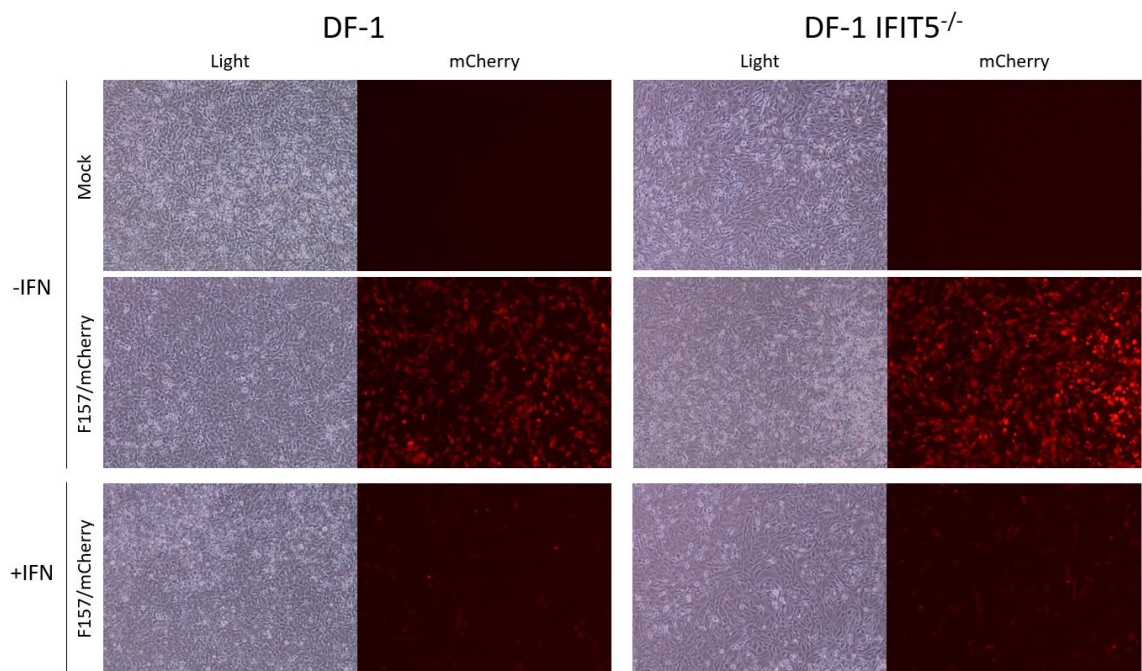


Figure 3.33 - Infection of DF-1 and DF-1 IFIT5^{-/-} cells with PIV5. Confluent cells were or were not pre-treated with IFN at 8h pre-infection, and then infected with PIV5/F157/mCherry at an moi of 10. At 18hpi, images were taken with a fluorescent microscope.

Vero cells lack type I interferon^{272,273} so the amount of IFIT1 induced during infection will be limited. Transfection of plasmids expressing an ISG into Vero cells can therefore show the effect of the ISG on viral infection in the absence of the other hundreds of ISGs induced by IFN. The MG system provides a model to study ISGs in isolation, but the presence of other viral proteins during infection could alter the sensitivity of the virus to an ISG. To check this, Vero cells were transfected with plasmids expressing either hIFIT1 or hIFIT1(R187H) from a bicistronic transcript which also encodes eGFP downstream of a poliovirus IRES sequence, so that cells which are positive for eGFP will also be positive for hIFIT1. Transfected cells were infected with PIV5/F157/mCherry at an moi of 5 and imaged at 18hpi (figure 3.34). Due to the low transfection efficiency of Vero cells, most cells were not positive for GFP, but for hIFIT1 all cells which were positive for GFP were also negative for mCherry, showing that hIFIT1 restricts viral translation. The images in 3.33 are representative of the whole population of cells in which no double-positive cell was able to be found. In contrast, for hIFIT1(R187H) numerous double-positive cells were found with only a slight reduction of mCherry intensity in these cells. These results mirror those with the MG in that the effect of hIFIT1 on PIV5 translation is dependent upon its ability to bind the 5' end of RNA (R187H disrupts this¹⁸⁰). An extension to this experiment would be to use other mCherry-expressing viruses, such as NDV, to infect transfected DF-1 cells to investigate the possibility that the virus recruits host MTases to methylate viral mRNA caps to evade restriction by IFITs. This idea will be expanded upon in the next chapter.

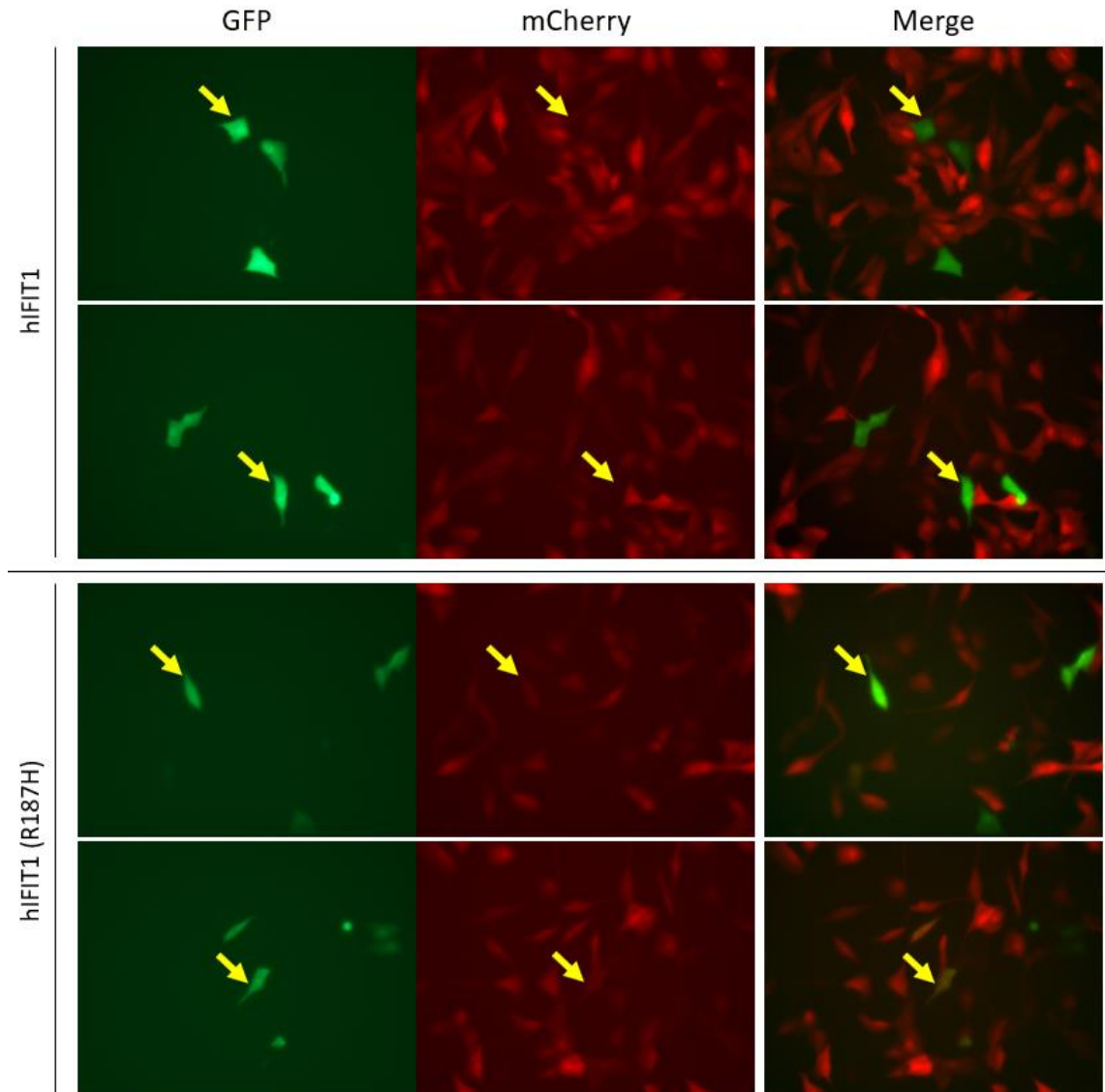


Figure 3.34 - Over-expression of hIFIT1 but not hIFIT1(R187H) inhibits PIV5 translation. Vero cells were transfected with pCAGGS-IFIT1/IRES.eGFP which expresses IFIT1 and GFP from a single transcript so that a GFP-positive cell will also be IFIT1-positive. At 30h post-transfection, cells were infected with PIV5/F157/mCherry at an moi of 5, and at 18hpi images were taken with a fluorescent microscope. Yellow arrows indicate examples of cells either solely positive for GFP (top) or positive for both GFP and mCherry (bottom).

3.4.3 Growth curve of PIV5 in A549 IFIT1^{-/-} cells

Finally, a growth curve of PIV5/F157/mCherry was performed in A549 cells. The impact of IFIT1 on PIV5 growth has not been fully investigated, with previous work using only IFIT1 knockdown cells and measuring viral titre at a single timepoint, or by measuring plaque size^{149,150}. PIV5/F157/mCherry was selected for this experiment because it allows for visualisation of plaques by expression of mCherry and the F157 change avoids the shutoff of viral transcription which may deemphasise any effect of IFIT1 on viral growth. The incorporation of mCherry into

the viral genome reduces virus-induced cytopathic effect (CPE) (Rick Randall, personal communication), but this disadvantage is overcome by detecting plaques by mCherry expression rather than relying on CPE and crystal violet staining of fixed cells.

A549 and A549 IFIT1^{-/-} cells were pre-treated with IFN for 8h and then infected with PIV5/F157/mCherry at an moi of 0.01. Cell supernatants were harvested at 12, 24, 48, and 72 hpi and the viral titre determined by plaque assay in Vero cells, with plaques visualised using a fluorescent microscope. The viral titres at each timepoint and condition are shown below in figure 3.35.

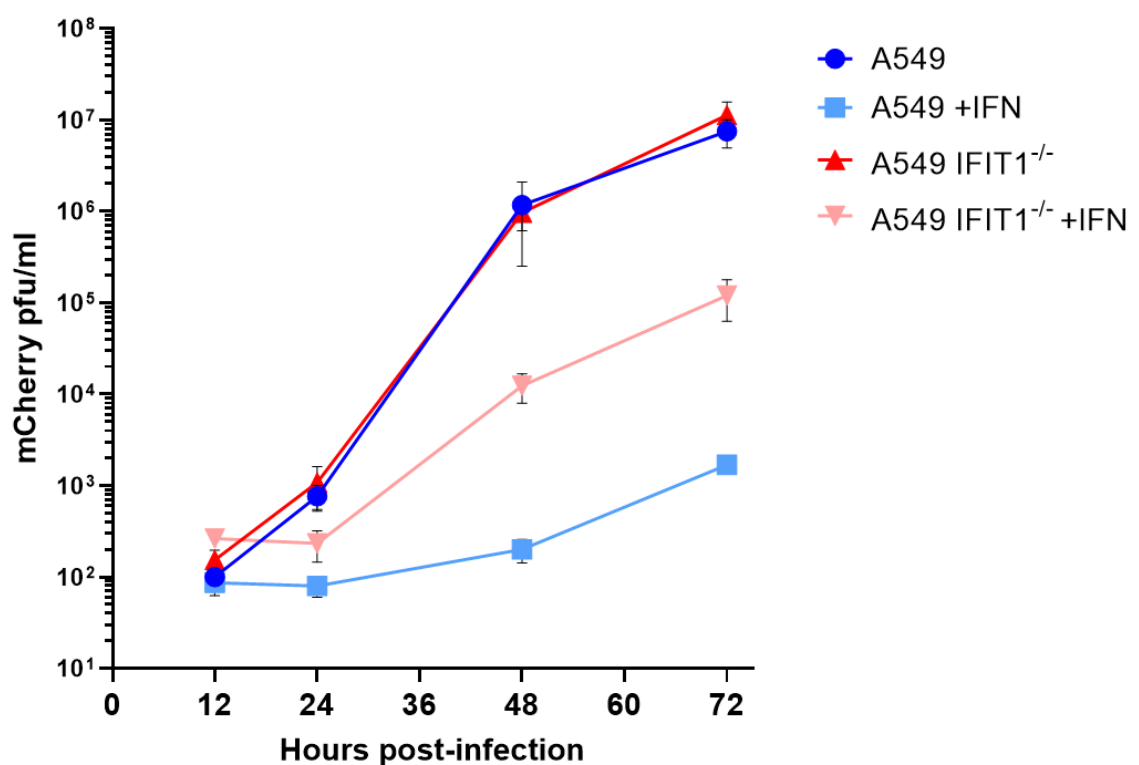


Figure 3.35 - Growth curve of PIV5 in A549 and A549 IFIT1^{-/-} cells. Confluent monolayers of cells were or were not pre-treated with IFN at 8h pre-infection, and then infected with PIV5/F157/mCherry at an moi of 0.01. Cell supernatants were harvested at 12, 24, 48, and 72 hpi and flash-frozen. Samples were titrated by plaque assay in Vero cells and results presented as pfu/ml. y-axis scale is logarithmic. Error bars are SEM and data are an average of three independent replicates.

The virus grew to a similar titre in both A549 and A549 IFIT1^{-/-} cells in the absence of IFN, showing that the virus likely doesn't induce IFN to any great extent during infection. This is corroborated by previous work which has shown that induction of IFN is a rare event during infection by negative sense ssRNA viruses¹²⁰. Pre-treatment of A549 cells with IFN resulted in a large decrease in viral titre at all timepoints from 24hpi onwards, with an approximate 1-log reduction at 24hpi, and a 4-log reduction at 48 and 72 hpi. Knockout of IFIT1 partially restored viral titres,

resulting in an approximate 2-log increase in viral titre at 48 and 72 hpi following IFN pre-treatment in A549 IFIT1^{-/-} cells relative to IFN pre-treated A549 cells. However, knockout of IFIT1 was not sufficient to completely recover viral growth to that seen in the absence of IFN, as the virus still grew to a titre approximately 2-logs lower at both 48 and 72 hpi. This contrasts with the results seen in figure 3.30, in which knockout of IFIT1 completely restored viral mCherry expression at 18hpi. Other ISGs are therefore likely important during PIV5 infection, acting on viral processes other than entry and initial transcription and translation. One possible candidate is tetherin, which prevents release of enveloped viral particles from infected cells^{140,287} and has been reported to inhibit the growth of rubulaviruses^{288,289}.

3.5 Chapter summary

In summary, PIV5 is sensitive to mRNA cap-binding IFIT proteins and this sensitivity is likely due to the deficient 2'-O-MTase activity of the L protein. IFIT1 is primarily, but not completely, responsible for the sensitivity of PIV5 to IFN in human cells, which raises the question of why an L protein deficient in 2'-O-MTase activity has evolved in rubulaviruses. Potential explanations for this will be expanded upon in the discussion section.

Chapter 4 – The differing sensitivity of PIV5 mRNAs to IFIT1

In chapter 3, it was shown that PIV5 is sensitive to mRNA-cap-binding IFIT proteins because it generates mRNA with a cap0 structure. Given that this structure will be present on the transcripts of all PIV5 genes, it might be expected that the levels of each PIV5 protein are equally sensitive to IFIT1. However, the degree to which each protein is affected differs greatly with NP, P, and V being much less sensitive than M and HN^{149,150,290}. Publications from the Randall group investigating the effect of IFN on PIV5 infection have repeatedly shown that while, for example, the level of P protein is reduced by about 40% by IFN, that of M is reduced by >90%²⁹⁰.

PIV5, like all *Mononegavirales*, produces separate transcripts encoding each viral protein. Each PIV5 transcript has unique 5' and 3' untranslated regions (5'UTR and 3'UTR)²⁸ and each gene is separated by intergenic sequences (IGSs) and regulatory sequences which modulate the levels of transcription initiation. The 5'UTR sequence proximal to the cap and the 5' flanking sequences recognised by the L protein are different for each PIV5 gene.

There are several possible explanations for the differing IFIT1 sensitivity of PIV5 genes:

- Some PIV5 transcripts may be more likely to have a cap1 structure than others due to differences in 5'UTR sequence which may differentially regulate L activity. This possibility is unlikely given the evidence in the previous chapter which shows that PIV5 generates cap0 structure on the MG mRNA (which has the NP 5'UTR).
- The 5'UTR sequence proximal to the cap may confer differential IFIT1 affinity on PIV5 mRNAs.
- The PIV5 proteins may show differences in stability which could exaggerate the effect of IFIT1 for proteins with a short half-life.
- A combination of the above possibilities.

This chapter attempts to delineate the mechanisms which determine the differential sensitivity of PIV5 genes to IFIT1, and to examine the consequences of this in terms of infection and viral fitness. To do this, the PIV5 5'UTR sequences are compared and their sensitivity to IFIT1 tested using MG reporters, RNA electrophoretic mobility shift assays (EMSAs), in vitro translation assays, and rescue of PIV5 viruses with mutated 5'UTRs.

4.1 The predicted RNA secondary structure of PIV5 5'UTRs

The differing sensitivity of PIV5 transcripts to IFIT1 is likely determined by the sequence of the 5'UTR. IFIT1 binding is not known to have any sequence-specificity because interactions in the

RNA-binding pocket are with the ribose of nucleotides rather than the base¹⁸⁰, making an explanation based simply on 5'-terminal primary sequence unlikely. However, RNA secondary structure at the 5' end of mRNAs has been reported to affect binding of IFIT1 in the case of alphaviruses. These are +ssRNA viruses of the *Togaviridae* family which lack a 2'-O-methyltransferase and generate genomes with cap0 structure^{206,208}. Differences in cytopathogenicity and IFN-sensitivity between strains of the alphavirus Venezuelan equine encephalitis virus (VEEV) have been mapped to a single nucleotide change at position 3 of the genome which disrupts a stable hairpin structure^{208,209,291,292}, the loss of which increases the affinity of IFIT1 for the capped VEEV genome²⁰⁸. Furthermore, binding studies of IFIT proteins using RNA oligomers have revealed differing affinity correlating with predicted RNA secondary structure despite identical 5' cap structure, as seen when comparing the unstructured human β -globin 5'UTR to that of the Zika virus genome²⁵³.

IFIT1 directly interacts with the ribose of the first 4 nucleotides of capped RNA¹⁸⁰, but toeprinting analyses (which are able to map protein binding to specific sequences by inhibition of reverse transcription) have shown that IFIT1 either interacts with or covers the first 7nt¹⁷⁹. This means that RNA secondary structure which includes base-pairing within the first 7nt, especially at the first 4 positions, is likely to reduce the ability of IFIT1 to interact with the 5' end. Therefore, the 5'UTRs of PIV5 transcripts were compared and predictions made of their secondary structure.

4.1.1 Paramyxovirus 5'UTR sequence alignments

The first 40 bases of the 5'UTR of PIV5 mRNAs were aligned and compared to those of other paramyxoviruses (figure 4.1). Although the first 2 bases (AG) are conserved across all PIV5 genes, the remainder of the 5'UTR is highly variable. This includes position 3 which is either a G (NP, P, HN, and L) or a C (M and F). This lack of conservation is also seen in the rubulaviruses PIV2 and MuV, though SV41 begins AGG for all genes. In contrast, the 5'UTRs of non-rubulavirus such as those of *Henipavirus*, *Morbillivirus*, and *Respirovirus*, maintain the sequence of the first 4, 3, and 4 bases, respectively. The genus with the most highly conserved 5' sequence is *Avulavirus* with, for example, the first 8 bases of NDV 5'UTRs being identical in all genes.

<u>Orthorubulavirus</u>		<u>Henipavirus</u>		<u>Respirovirus</u>	
PIV5		HeV	SeV		
NP	AGGTCGGAAACCTATGGCCTTCGTGACCGACCTCGAGTCA	NP	AGGAACCAAGACAGTACAAATGGTCTTGGTATTGGACAA	NP	AGGGTCAAAAGTATCCACCTCGAGGACAGGTTCCAGACCC
P	AGGCCCGAGCGGTTAGCAACACGCGACTGCCGTGGCCAA	P	AGGATCCAAAGACATAAATCTAGGATCCTTTACAAATCTCA	P	AGGGTCAAAAGTTCATCCACCGATCGGTCCAGCAAGGCCA
M	AGCCGAAACACTACACTACCTACCTCAGGACGCGCATGCCATC	M	AGGAGACAGGTATAATATTTCTACACCTGTCCATTCACCT	M	AGGGTCAAAAGAAATTTTCACTAACCGCGGCACATGGCAGA
F	AGCAGAACCCATATCGTCCCTCAAATCATGGGTACTATA	F	AGGAGCCAAAGTTTTGGTCTCTTTAGATTAAATCAAGCTGTT	F	AGGGATAAAGTCCCTTCTGAGTGTGGTTCGAAAACCTCT
HN	AGGCCGGAACACTAATCGTCCGCGCAGTGCCTGCACAC	HN	AGGACCCAAAGTCTTAAACCACTTCTAATGTGAGGGAGAA	HN	AGGGTCAAAATGAGGTCCGCGGTACTTTAGCTTTTCACCT
L	AGGCCAGAAATGSGCTGGGTCTCGGGAGATATTACTCCCTGA	L	AGGACCCCAAGTCTCTTTTAAACGTCATGATTGAGATAGTCA	L	AGGGTCAATGGGAAGCTTGCATAGGTCAATGATGGCGAG
PIV2		NiV	PIV3		
NP	AGATTCTGGTCCGGTAAACGATTCACAGTTTATACTATCTG	NP	AGGAACCAAGACACAAACACTTTTGGTCTTGGTATTGGATCC	NP	AGGATTTAAGACATTTGACTAGAAAGGTCAAGAAAAGGGAAC
P	AGGCCGAGCGGTTAGAAATCCGGTGTCTTCTGTGATCA	P	AGGATCCAAAGATTTACTCTAGGATCCTCTATTAAAGCTT	P	AGGATTTAAGAAATCCTATTATACCAAGATATAGGTGGTA
M	AGGTCGGAAGCTTGCTCTCAGACAGATCCCAAAATCAT	M	AGGAGACAGGTATAGAACCTCTCTTTCAGATAACTGGTCA	M	AGGATTTAAGAAATAAATAATTAATCTTATCCAAAATAGGATAT
F	AGGCCAAAATATTACGCCATGTCATCAGTCCCAATGA	F	AGGAGCCAAAGCTCTTCCCTCGTTCAGAAAGGTTAAACAAGC	F	AGGACAAAAGAGGTCAACACCAACCACTATTAGCAGTCAAT
HN	AGCAGAACCCCTTAGGTGTGTAACGTCGTGCACACC	HN	AGGACCCAGTCCATACACTCATTTGATTAACACTATCTGATC	HN	AGGATTTAAGTTACGCAATCCAACTCTACTCATCAATTTG
L	AGGCCAGAAATGSGCGGCTCTTCTGAGATACCTCCTTCTCTGA	L	AGGACCCCAAGTCTCTTGATTATGCCAATTTTCTCGAGAAAT	L	AGGAGCCAAAGCGTCTCGGAAATATGACACTGAATCTTAAC
MuV		<u>Morbillivirus</u>		<u>Orthoavulavirus / Metaavulavirus</u>	
NP	AGGCCCGGAAGTGGTGTGTTTTCGGATTTCGAGGCGGGGCTC	MV	AGGATTCAGATCCTATTATCAGGACAAAGACAGGATTA	NP	ACGGGTAGAAAGGTGTGAATCTCGAGTCCGAGCCCGAAGCA
P	AGGCCCGGAAGAAATTAGTTCTACGAGCATGCACAAAT	P	AGGAACAGGTCCACACAGCGCCGACCAACCAACCATCC	P	ACGGGTAGAAAGAGGATATTTCAGAGATCAGGGCAAGTCTC
M	AGGACGAAACAAAGTCCCAATCCCAACCATAGCAGCAATGG	M	AGGAGCAAGTGTATTGCTCTCTAAGTTCCACATGACAGA	M	ACGGGTAGAAATTTGAGTCCCAATTTGTCCTCAAGATGGAC
F	AGGCTTAGAGGATATCTTACTTCTGACTTCCCAACTTC	F	AGGCCCAAGGAACACACACTCGACACAAACCCGACCCC	F	ACGGGTAGAAAGACTCTGTGATCCCGGTTCGGCGCTCCAGG
HN	AGGCCAGAACAGCTTAGGATCACAATACACACAGAAC	HN	AGGTCGCAAGATCATCCACATGTCAACACACGAGACCG	HN	ACGGGTAGAAAGCGTAAAGAGAGCGCCCTCAATTGCGAG
L	AGGCCAGAAATGCGCGGCTAAATGAGATACTCTTACCTGA	L	AGGTCCAAGTGTGTTCCCGTTATGGACTCGCTATCTGTC	L	ACGGGTAGGAATGCGCGAGCTCCGGTCTCTGAAAGGCGAGA
SV41		CDV	APMV6		
NP	AGGCCCGGAACACAGTGTGCTCCGGTGGCGGTTGCGATTTCG	NP	AGGGTCAATATGATCCTACTTAAGAAACAAAGGTAGGGTTC	NP	AGGGGGAAGCTGTGGACTCCGGGTCCGGAGTCCGTTGGAC
P	AGGCCCGGACTGGTATCACACCCCGCGGTCTTTCAAGGCT	P	AGGACCAAGTCTCAACAAACCCGATCAATCATTCATCCGA	P	AGGGGGAAGTCTTCTGGTGGCGGGCTAGGCCCATATGAC
M	AGGTCGGAACCCAGGACAAACCGCCACACACCCACCGAC	M	AGGACCAAGAGCCTAAGTCCGCTCTCTAAAATATGACTGA	M	AGGGGGAAGTCTGTTGTTCTCGGACTGGGCAACCCCAAGTTC
F	AGGACAGAACCCCTTAGGTGGCGGTAAGTCTGCTGGGCACT	F	AGGTCAGACATAGCAAGCAACAGTCAACAGGTCAACAGTCC	F	AGGGGGAAGGCAATGGGAGCCCACTGGGCGCTCTTAGCAAT
HN	AGGCCAGAAATGCGCGGCTCTGCTGACATCTCTGTTGCCCGA	HN	AGGGCTCAGTAGTCCAGCAATGTCTCCCTACCAACAGCA	HN	AGGGGGAAGGAGGTTGACTTCGCACTCAATTGATCAGAG
L		L	AGGATCCAGATCCTTTTAGCCATGGACTCTCTATCAGTG	L	AGGGGGAAGGCAGGCTGGGATCACATCCAGCTGTAGCTT

Figure 4.1 - 5'UTR alignments of genes from representatives of Paramyxoviridae genera. The first 40 bases of each of the conserved 6 paramyxovirus genes (NP, P, M, F, HN, and L) are shown with bases conserved across at least 5 of the particular virus's genes highlighted in yellow. For consistency, NP and HN are used despite varying nomenclature (i.e. N and G). P is used to represent all P/V/C/W transcripts. When the start codon of the CDS is present in the first 40 bases, it is shown in bold. Full virus names can be found in the abbreviations section. GenBank accession numbers for the aligned virus genomes can be found in the methods (section 2.5).

The increased gene-specific variation of *Rubulavirus* 5'UTRs raises the possibility that these sequences are under a selective pressure not exerted on the 5'UTRs of other paramyxovirus genes. Given the established sensitivity of rubulaviruses – and the insensitivity of non-rubulaviruses – to IFIT1, this 5'UTR variation is possibly reflective of the need to evade IFIT1 binding in the absence of cap1 mRNA structure. Notably, although NDV has been reported to produce cap0 mRNA²⁵⁴, chickens lack a canonical cap0-binding protein^{161,245} and thus NDV 5'UTRs would not be under the same selective pressure as those of PIV5, possibly explaining the lack of diversity shown in figure 4.1.

4.1.2 PIV5 5'UTR secondary structure predictions

To assess whether RNA secondary structure differs between PIV5 5'UTRs, the first 40 bases of each transcript were submitted to an online RNA secondary structure prediction program (Vienna RNAfold WebServer²⁹³). The predicted structures are shown in figure 4.2 with the location of the cap at the 5' end indicated. The same sequences are shown below in dot-bracket notation along with the minimum free energy (MFE) value of each prediction.

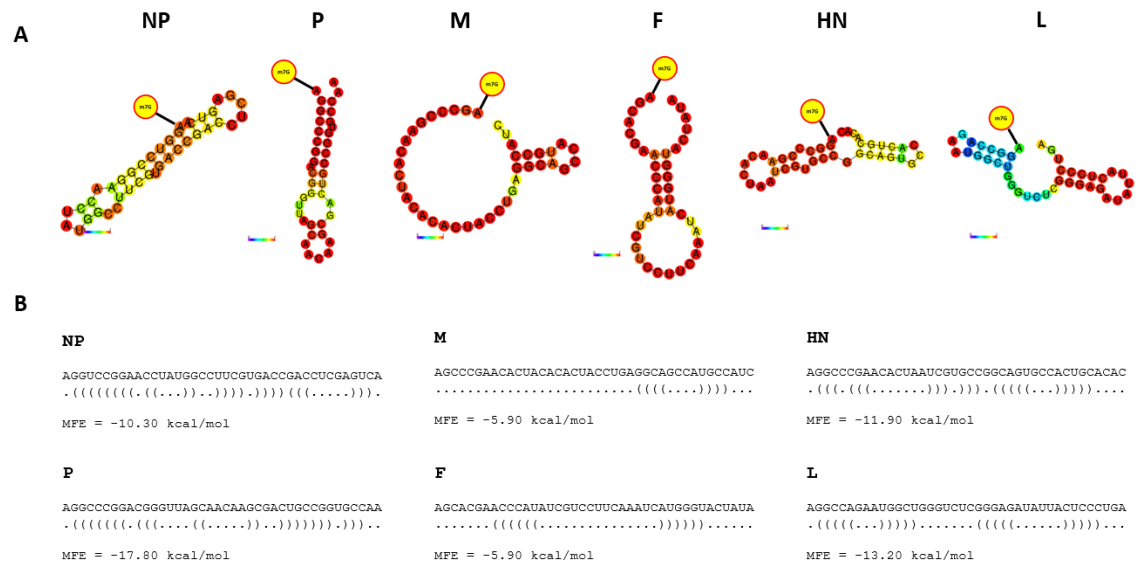


Figure 4.2 - RNA secondary structure predictions for the first 40 bases of each PIV5 (W3A) transcript. A) Minimum free energy (MFE) structure prediction diagrams with base-pairing probability shown with red being highly probable and purple being uncertain. The location of the m7G cap is indicated. B) The sequence of the above diagrams shown in dot-bracket notation and with the MFE value. Structure predictions were made using the ViennaRNA RNAfold Server with the default parameters.

Both the NP and P 5'UTRs are predicted to form long hairpin structures which leave a 5' overhang of just 1 unpaired base. In particular, the structure of the P 5'UTR is predicted with high probability (as indicated by the red-coloured bases in the diagram) and with a low MFE. In stark contrast, the 5'UTRs of the M and F genes are predicted with high probability to form no secondary structure at the 5' end, resulting in 5' overhangs of 26 and 7 unpaired bases, respectively. Since IFIT1 binding is known to cover the first 4-7 nt¹⁷⁹, the downstream hairpin structures of the M and F 5'UTRs would not be expected to have an impact on protein binding, in contrast to the 5'-terminal structures of the NP and P 5'UTRs. The predicted structures of the 5'UTRs of the HN and L genes gave intermediate results with both having a 1-base overhang but with either a much shorter hairpin structure than the NP or P 5'UTRs (for HN) or a structure with low predicted probability (for L). All of these results were corroborated by submitting the same sequences to a different online prediction program (RNAstructure²⁹⁴) from which the same predicted structures were obtained (data not shown).

Using just the first 40 bases of each viral mRNA for secondary structure predictions could give results which are not relevant to the full-length mRNA in cells. This is because 1) downstream sequences may promote the formation of different structures at the 5' end, and 2) RNA folding occurs co-transcriptionally as the molecule is released by the polymerase and this may result in different structures forming compared to, for example, those formed when the full-length mRNA is heat-refolded^{295,296}. To address this issue, the full-length sequences of the PIV5 mRNAs were submitted to the online CoFold program²⁹⁷ which provides structure predictions which take co-transcriptional folding into account.

The first 100 bases of the CoFold predictions for each gene are shown in figure 4.3 in dot-bracket notation. Where local 5' structures are predicted to form, the bases involved have been highlighted in yellow. Both the NP and P 5'UTRs are predicted to form the same tight hairpin structures shown in figure 4.2 which leave just a 1-base 5' overhang. Similarly, both the M and F 5'UTRs are still predicted to have long unpaired 5' overhangs. Although some additional structure is predicted for M via pairing with sequences downstream of the first 100 bases, this still leaves an 18-base 5' overhang which should not differ from the previously predicted 26-base overhang in terms of IFIT1 binding. However, the full-length prediction of both the HN and L 5'UTRs differ compared to the 40-base predictions shown in figure 4.2. The HN 5'UTR is now predicted to form a 5' hairpin binding to sequences at positions 89-97, while the L 5'UTR is predicted to form 5' structure binding to sequences downstream of the first 100 bases and thus not shown in figure 4.3. Accuracy of RNA folding prediction decreases with increasing distance between the paired bases²⁹⁸⁻³⁰⁰, so the long-range structures predicted for the HN and L 5'UTRs are likely to be of lower probability than the short local hairpins formed by the NP and P 5'UTRs.

targeting bases downstream (i.e. more than 7nt) from the 5' end, the predicted structure can be disrupted while maintaining the same 5'-proximal sequence. This is important because it excludes any possible sequence-specificity of IFIT1 and maintains the viral gene start signal, which should prevent any changes to viral transcription and MTase activity when these mutations are incorporated into the virus in later sections. P and M were chosen to be the primary focus because they have the strongest and weakest predicted structures, respectively; there are antibodies available against these proteins; and the varying levels of these two proteins are likely to produce differences in viral phenotype (discussed at length later).

The bases at positions 2-4 (GGC) of the P 5'UTR are predicted to pair with bases 36-38 (GCC) (see figures 4.2 and 4.3). To disrupt the 5' hairpin structure, bases 36-38 were mutated to adenines and the new sequence submitted to the RNAfold server as before. This new 5'UTR, P(mut1), is now predicted to have a 4-base 5' overhang (figure 4.4). It proved hard to introduce changes which disrupt the predicted 5' structure of both P and NP because different conformations were predicted to form even when disrupting the original predicted structure. Although P(mut1) has a 5' overhang, many other attempted changes simply formed new predicted structures without a substantial 5' overhang. This is perhaps reflective of the high GC content of the first 40 bases of both P (65%) and NP (60%) which may promote formation of strong secondary structure. Therefore, only a single mutant was designed for P.

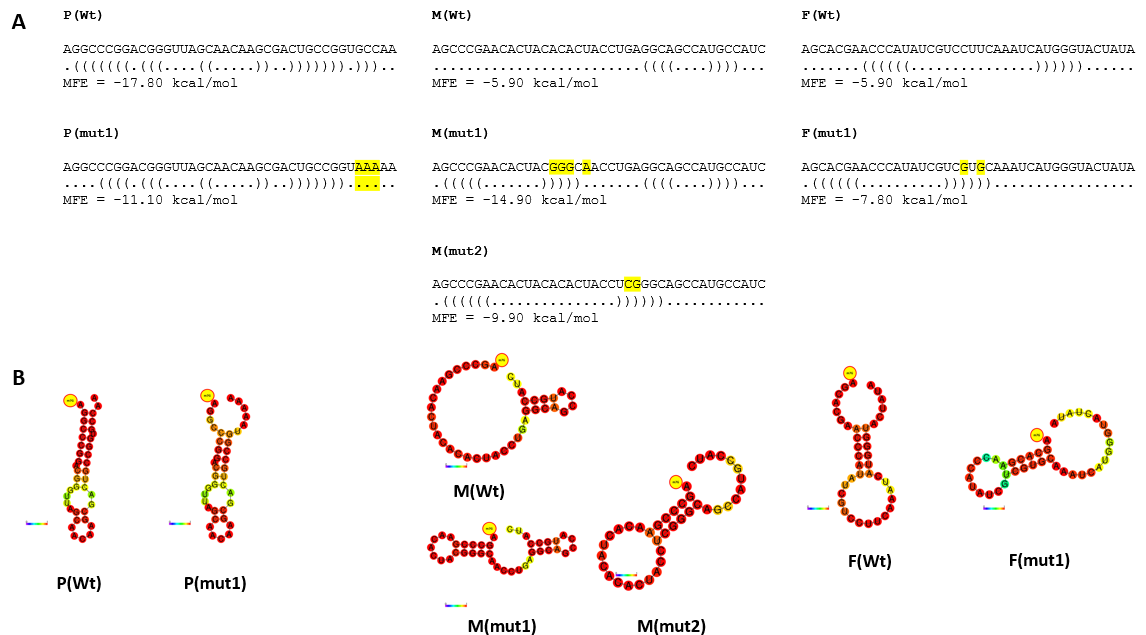


Figure 4.4 - Mutations to change the predicted secondary structure of PIV5 5'UTRs. A) Highlighted in yellow are mutations introduced downstream from the 5' end of the transcript which are intended to destroy (P mutants) or introduce (M and F mutants) hairpin structures proximal to the 5' end. The new predicted MFE structures are shown in dot-bracket notation. Predictions were made as before using the RNAfold Server. **B)** Predicted MFE structure diagrams of the above sequences with the probabilities shown as before.

In contrast, it was simple to introduce predicted secondary structure for both M and F. This was done by changing downstream sequences so that they are complementary to bases at the 5' end. For M(mut1), bases 14-19 (CACACU) were changed to CGGGCA so that they would be complementary to the GCCCG sequence at bases 2-6. A second mutated M 5'UTR, M(mut2), was made by changing bases 24-25 (GA) to CG so that the new UCGGC sequence is complementary to the GCCCGA sequence at bases 2-7. Finally, a mutated F 5'UTR, F(mut1), was made by changing bases 20-22 from CUU to GUG so that the new UCGUG sequence is complementary to the GCACGA sequence at bases 2-6. These changes to the M and F 5'UTRs all are predicted to introduce hairpin structures at the 5' end with a 1-base unpaired overhang (figure 4.4).

The new predicted structures for M and F were all designed to have a 1-base overhang. Although removing the overhang (i.e. by base-pairing the 5'-terminal base) might be expected to have a maximal effect on IFIT1 binding, it is notable that none of the predicted structures for PIV5 5'UTRs with 5' structure have a blunt base-paired 5' end (see NP and P in figures 4.2 and 4.3); instead, a 1-base overhang is always present. The overhang may be required to maintain binding to translation factors³⁰⁴ or to prevent formation of a PAMP detected by RIG-I, which recognises ppp-RNA with a base-paired 5'terminal base^{101,305} and which can accommodate cap0 structures (but not cap1) in its RNA-binding pocket^{276,277}. Thus, the designed 5'UTRs above can be used not only for in vitro binding analyses, but also for experiments which would be affected by failure to recruit translation factors or by RIG-I-dependent induction of IFN by viral mRNA.

Having designed these 5'UTRs, their relative sensitivities to IFIT1 were tested in the following sections by MG assay, EMSA, in vitro translation, and western blot of infected cells.

4.2 Bicistronic PIV5 MGs with variable 5'UTRs

The PIV5 MG plasmid from the previous chapter uses the 5'UTR of NP so that the first 101 bases of the reporter gene mRNA are of viral origin. This viral sequence also contains elements of the bipartite promoter required for replication³⁰⁶. Specifically, three CG dinucleotides at positions 22-23, 28-29, and 34-35 of the NP 5'UTR are absolutely required for promoter activity and cannot be changed. As a result, it is not possible to simply swap the 5'UTR of the MG to that of other PIV5 genes as a way to investigate their differing sensitivity to IFIT1.

Therefore, a Bicistronic MG reporter (BiMG) was designed to incorporate the first 40 bases of the NP, P, M, and F 5'UTRs. The BiMG consists of a Renilla luciferase (Rluc) reporter gene as the first cistron with the viral Le and NP 5'UTR sequences upstream of the start codon, as in the

standard MG. Downstream of the Rluc stop codon, a variable section of viral sequence is introduced consisting of a 3'UTR, and IGS, and a 5'UTR. This is followed by the CDS of Fluc, and the sequences following the Fluc stop codon (L 3'UTR and Tr) are the same as in the standard MG. The sequence between the Rluc and Fluc CDSs is dependent upon the 5'UTR being investigated. For example, this region of BiMG-P40 contains the NP 3'UTR followed by the NP/P IGS followed by the first 40 bases of the P 5'UTR. BiMG-M40 contains the P 3'UTR, the P/M IGS, and the first 40 bases of the M mRNA. Maintaining the correctly matched 3'UTR, IGS, and 5'UTR sequences as found in the viral genome appears to be important because an attempt at making BiMGs for the M and F 5'UTRs with a mismatched upstream 3'UTR and IGS (the NP 3'UTR and the NP/P IGS) failed to produce any Fluc reporter (data not shown).

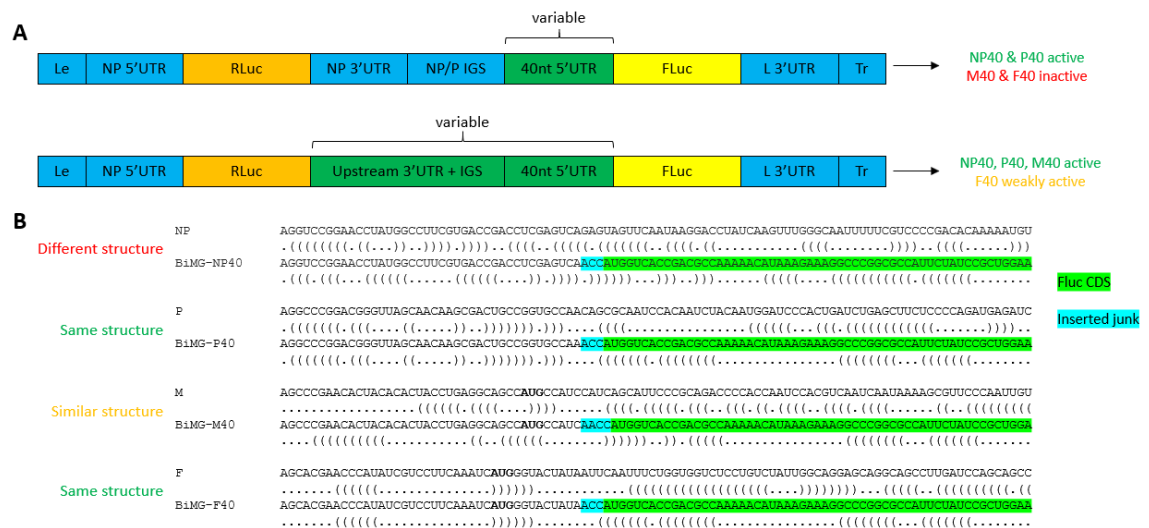


Figure 4.5 - Construction of bicistronic PIV5 minigenomes. A) Diagrams of BiMG variations. Attempted construction of BiMGs with the NP 3'UTR and the NP/P intergenic sequence (IGS) (top) resulted in Fluc expression when joined to either the NP40 or P40 5'UTRs, but not when using other 5'UTRs. Matching the upstream 3'UTR and IGS to the 5'UTR in use (bottom) resulted in Fluc expression for all 5'UTRs. The NP40 5'UTR is paired with an upstream NP3'UTR and NP/P IGS. **B)** Co-transcriptional folding predictions were made using CoFold as before, with the first 100 bases shown. The predicted 5'-proximal structure for each viral transcript (e.g. P) is compared to the predicted structure when the first 40 bases are fused to the Fluc CDS (e.g. P40-Fluc). The Fluc CDS is shown in green. Sequence introduced to maintain the rule-of-six, reading frame (for M and F which have start codons upstream of the Fluc start codon, shown in bold), and a constant Zokak sequence is shown in blue.

The length of the 5'UTR introduced into the BiMG constructs was maintained at 40 bases to be consistent with the RNA secondary structure predictions made in the previous section. This means that the 5'UTR of BiMG-P40 is truncated relative to the viral transcript, and the 5'UTR of BiMG-M40 is extended because the start codon of M is at positions 33-35. Between the 40-base viral 5'UTR and the start codon of Fluc, a short non-viral sequence ($A_{(n)}CC$) of variable length was introduced to conform to the 'rule of six', to maintain a constant Kozak sequence, and – in the

case of BiMG-M40 – to ensure that the start codon at positions 33-35 is in frame with the start codon of Fluc. A schematic of the BiMG is shown in figure 4.5A.

To confirm that similar 5' structure is predicted when the Fluc CDS is included downstream of the viral 5'UTRs, the entire Fluc mRNA produced by each BiMG was submitted to the CoFold program, as before. Both BiMG-P40 and BiMG-F40 are predicted to form the same structure. However, the predicted BiMG-NP40 structure has a completely different 5' hairpin which is much shorter and pairs further downstream. BiMG-M40 also has a different predicted structure with a shorter 4-base overhang, but the hairpin formed here is predicted to base-pair with a sequence much further downstream (positions 420-429 not shown in figure 4.5) which, as previously discussed, is likely of low probability. The BiMG-P40 and BiMG-M40 were selected for further investigation as their predicted structures are the same or similar to those predicted to be formed in the viral mRNAs. BiMG-NP40 was excluded because of the different predicted structure, as was BiMG-F40 because of its low activity relative to the other BiMGs (data not shown).

Both the BiMG-P40 and BiMG-M40 reporters produced good S/B ratios, albeit typically lower than the standard PIV5 MG (data not shown). The longer minigenome RNA may require more NP to be encapsidated but using the previously optimised amounts of helper plasmids produced a good level of activity so was not further adjusted. Bicistronic minigenomes can be used to investigate the relative importance of viral UTRs³⁰⁷, for example by normalising the level of one reporter protein with that of a constant one (e.g. Fluc and Rluc, respectively), but this feature was not utilised in this project.

4.2.1 Comparison of PIV5 5'UTR sensitivity to IFITs using the BiMG system

The sensitivity of the BiMG-P40 and BiMG-M40 reporters to hIFIT1 and mIFIT1 were compared (figure 4.6). Strikingly, BiMG-P40 was completely insensitive to 125ng of transfected pEFV5-hIFIT1 while BiMG-M40 was highly sensitive. The unexpected increase of BiMG-P40 signal by hIFIT1 is reminiscent of the increased signal of the MV MG by IFIT1 seen in the previous chapter and implies that below a certain threshold of affinity or concentration, IFIT1 can have a stimulatory effect on viral protein production. As seen with the standard PIV5 MG in the previous chapter, mIFIT1 has a much stronger inhibitory effect than hIFIT1 and this result is repeated here with BiMG-P40 signal being reduced to >10% by 125ng of pEFV5-mIFIT1. Again, BiMG-M40 was more sensitive to mIFIT1 with no signal being detected at 125ng.

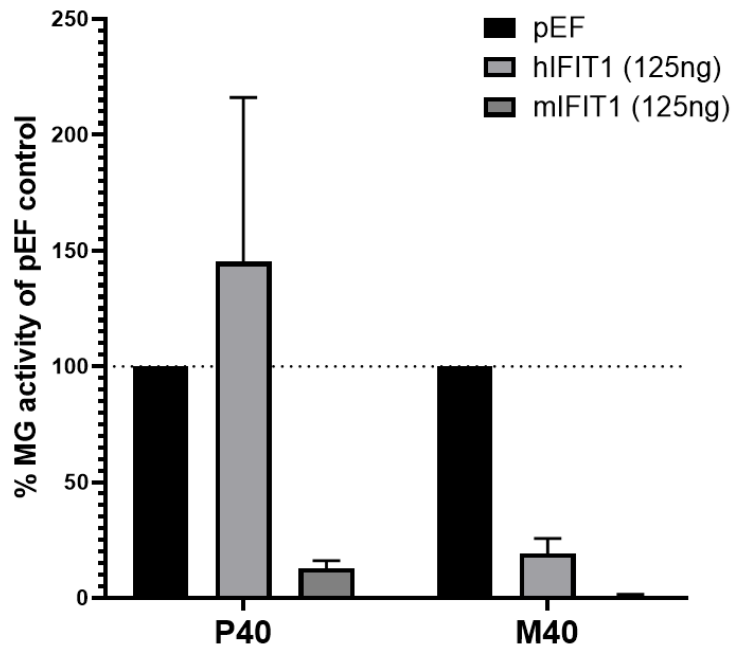


Figure 4.6 - The sensitivity of P40 and M40 BiMGs to hIFIT1 and mIFIT1. The BiMG plasmids were transfected into 293 IFIT1^{-/-} cells with the other MG system plasmids, as before, along with the indicated amount of pEF, pEFV5-hIFIT1, or pEFV5-mIFIT1 per well of a 12-well plate. Error bars are SEM and data are an average of at least three independent replicates.

The results with BiMG-P40 and BiMG-M40 show a clear difference in IFIT1 sensitivity between the P 5'UTR and the M 5'UTR. Because the encoded protein is the same (Firefly luciferase), this excludes differences in protein half-life explaining the differing sensitivity of P and M production during PIV5 infection and shows that the differing sequence of the 5'UTRs must be responsible. However, it is not possible to conclude whether the IFIT1 sensitivity is due to differing cap methylation efficiency, sequence specificity of IFIT1, or differing 5' RNA secondary structure. To test these remaining possibilities, mutated P and M BiMGs were made which incorporate the designed downstream mutations shown previously in figure 4.4. As before, the new BiMG Fluc transcripts were submitted to the CoFold program and the predicted structures are shown in figure 4.7A. Unlike the prediction for the 40mer shown in figure 4.4, here BiMG-P40(mut1) is still predicted to form a structure with a 1-base overhang, despite the GCC to AAA change downstream, by folding into a new structure. This difficulty in designing a P 5'UTR with disrupted structure was previously discussed in section 4.1.3. However, the new structure is predicted to form a shorter hairpin than the unchanged BiMG-P40, and the base pairs at positions 2 and 3 are now both G:U rather than the stronger G:C pair. Therefore, BiMG-P40(mut1) is likely to have weaker 5' structure and should be more sensitive to IFIT1 if secondary structure is indeed the main determinant of sensitivity. In contrast, the BiMG-M40(mut1) and BiMG-M40(mut2) predictions match those of the 40mers in figure 4.4, and therefore form much stronger

predicted 5' structure than the unchanged BiMG-M40, regardless of whether the distantly base-paired structure shown in figure 4.5 forms or not.

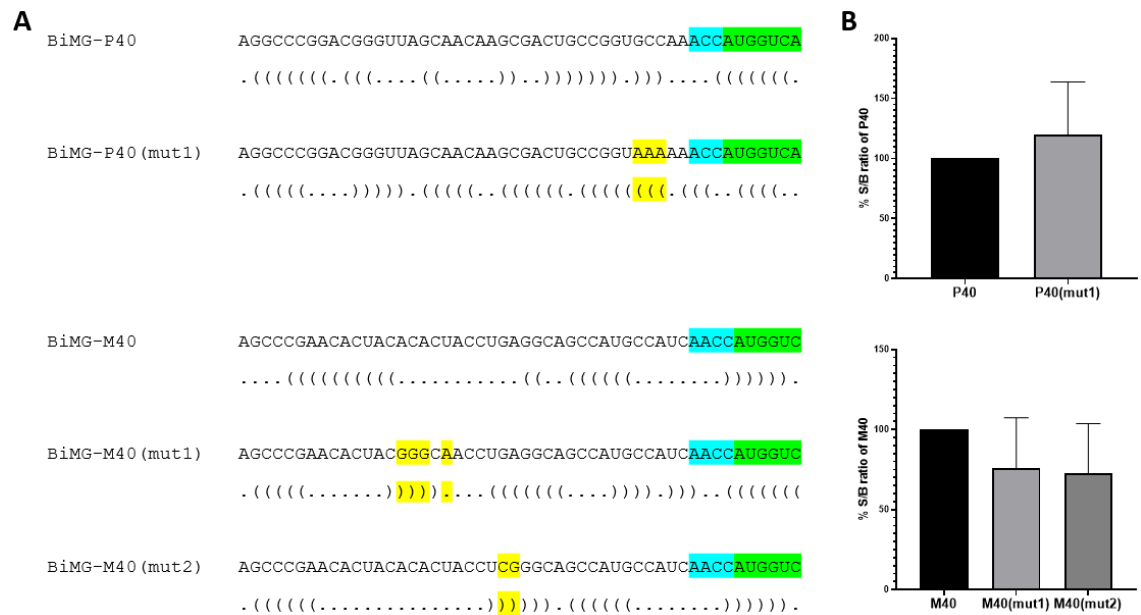


Figure 4.7 - BiMGs with mutated 5'UTRs. A) Co-transcriptional folding predictions for BiMG-P40 (top) and BiMG-M40 (bottom) along with the indicated mutated BiMGs. Changes to the 5'UTRs are shown in yellow; inserted junk sequence is in blue; the Fluc CDS is in green. **B)** Functionality of the mutated BiMGs as shown by the percentage of the S/B ratio of Firefly luciferase relative to the unedited P40 or M40 BiMGs. Error bars are SEM and data are an average of at least three independent replicates.

The activity levels of these mutated BiMGs were similar to those of the unchanged BiMGs, as shown in figure 4.7B. This shows that downstream mutations in the 5'UTRs have no major effect on viral transcription and that these changes can be incorporated into the virus (see later sections). Although these BiMGs are not designed to investigate differences in translation efficiency between reporters, it was noticed that the predicted 5' secondary structure had a weak negative correlation with S/B ratio; disrupting the P40 structure increased the S/B ratio while introducing structure to M40 decreased it. This could be due to secondary structure reducing the binding of translation factors such as eIF4F and eIF4G³⁰⁴, but this experiment is not properly controlled to investigate this so no strong conclusions can be made.

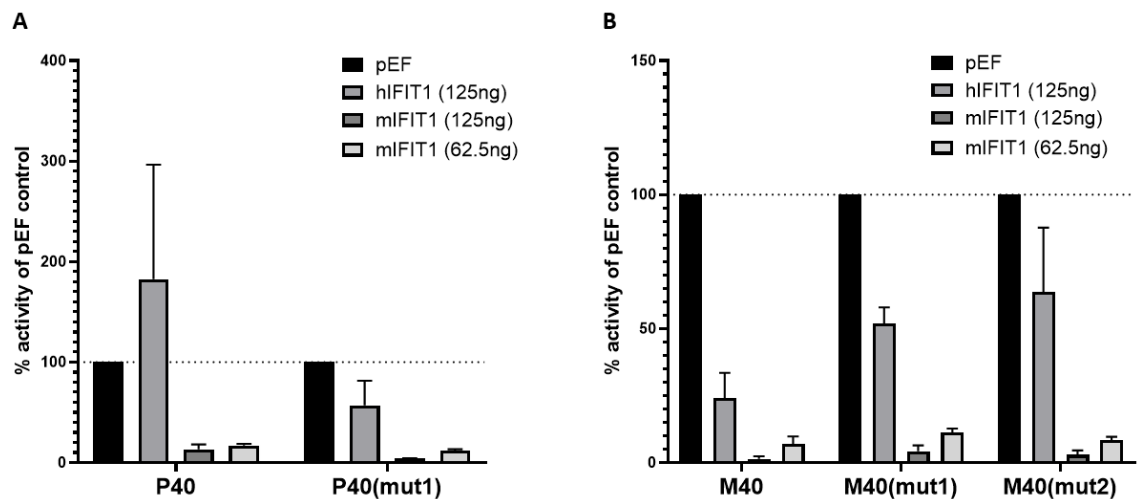


Figure 4.8 - The sensitivity of P40 (A) and M40 (B) mutated BiMGs to hIFIT1 and mIFIT1. The BiMG plasmids were transfected into 293 IFIT1^{-/-} cells with the other MG system plasmids, as before, along with the indicated amount of pEF, pEFV5-hIFIT1, or pEFV5-mIFIT1 per well of a 12-well plate. Error bars are SEM and data are an average of at least three independent replicates.

The mutated BiMG-P40 was tested with hIFIT1 and mIFIT1, as before. BiMG-P40(mut1) showed greatly increased sensitivity to hIFIT1 while the small signal seen with the unchanged BiMG-P40 at 125ng pEFV5-mIFIT1 was completely lost (figure 4.8A). These differences in sensitivity were clear despite BiMG-P40(mut1) still being predicted to form a 5' hairpin, albeit one weaker than P40. The opposite effect was seen with the M40 mutants (figure 4.8B), with both BiMG-M40(mut1) and BiMG-M40(mut2) showing increased resistance to hIFIT1 and some level of detectable signal at 125ng pEFV5-mIFIT1, in contrast to the unchanged BiMG-M40 which was totally inhibited.

The effect of the downstream mutations is strong evidence for RNA secondary structure determining the relative sensitivity of PIV5 mRNAs to IFIT1. These changes cannot directly impact the binding site of IFIT1 (the first 4-7nt^{179,180}) and are unlikely to affect recognition by the L protein either in terms of transcription initiation or cap methylation, both of which are regulated by the 5'-terminal RNA sequence of each gene^{28,198,308,309}. Although a role for small differences in cap1 methylation between P and M cannot be excluded, the clear correlation between predicted secondary structure and sensitivity to IFIT1 suggests a model in which PIV5 transcripts are all cap0 but differ in their 5' structure, which results in differing affinities for IFIT1. The in vitro affinity experiments in the next sections remove the potentially confounding factor of differing methylation status, while incorporating the UTR mutations into the virus itself at the end of this chapter confirms that the predicted structure differences are relevant during infection.

4.3 In vitro assays to test the affinity of IFIT1 for PIV5 5'UTRs

The non-viral sequence downstream of the 5'UTR in the BiMG (i.e. the Fluc CDS) may disrupt the 'authentic' RNA secondary structure present in the virus. It is therefore desirable to eliminate this possibility by studying the affinity of IFIT1 for the 5'UTRs using in vitro binding assays. RNA electrophoretic mobility shift assays (EMSAs) using short labelled RNA probes and purified recombinant protein can quantitatively demonstrate the affinity of a protein for an RNA molecule by visualising the change in electrophoretic mobility (the 'shift') of the RNA probe following binding (or not) of the protein at various concentrations. EMSAs have an advantage over other methods, such as toeprinting assays^{310–312}, in that the relative size of the complex(es) formed on the RNA can be detected, which is of relevance to IFIT proteins which are known to oligomerise^{162,163,179,244,253}. Toeprinting assays, which rely on inhibition of reverse transcription by the binding of proteins to RNA, have an advantage over EMSAs in that they can identify the specific nucleotides that are bound by the protein. However, the binding of IFIT1 to the 5' end of RNA has already been established and the current study is investigating the relative affinity for differing RNA probes rather than the binding mechanism of the protein.

The 5' 40 bases of the NP, P, M, and F 5'UTRs were selected for analysis by EMSA with purified recombinant hIFIT1 and mIFIT1. The mutations in the P and M 5'UTRs previously described were also included in this analysis, as well as one for F.

4.3.1 Generation of fluorescently-labelled RNA probes for EMSA

EMSAs require the production of RNA probes which are short enough to be clearly 'shifted' by protein binding and which are labelled to enable imaging. The production of probes for this study was informed by a published EMSA with IFIT1¹⁸⁰ which used 43-mer RNA produced by T7 polymerase in vitro transcription (T7-IVT) and labelled at the 3' end (by T4 ssRNA RNA ligase) with pCp-Cy5, a commercially available nucleotide modified with the fluorophore Cy5 (excitation peak at 651nm and emission peak at 670nm). T7-IVT produces uncapped triphosphorylated RNA (ppp-RNA) to which IFIT1 binds only weakly and is not relevant to the study of capped mRNA. Therefore, the RNA probes must be capped to m7GpppN which previous studies have achieved by using commercially available capping enzymes, typically Vaccinia capping enzyme^{174,178,180,208,244,253}.

The approach outlined above was used to generate RNA probes for the following sequences with the non-viral sequence at the 3' end underlined and mutated bases in bold:

NP40 (Wt)	AGGUCCGGAACCUAUGGCCUUCGUGACCGACCUCGAGUCA <u>AAAAAC</u> -Cy5
P40 (Wt)	AGGCCCGGACGGGUUAGCAACAAGCGACUGCCGGUGCC <u>AAAAAC</u> -Cy5
P40 (mut1)	AGGCCCGGACGGGUUAGCAACAAGCGACUGCCGGU AAA <u>AAAAAC</u> -Cy5
M40 (Wt)	AGCCCGAACACUACACACUACCUGAGGCAGCCAUGCCAUC <u>AAAAAC</u> -Cy5
M40 (mut1)	AGCCCGAACACUAC GGGCA ACCUGAGGCAGCCAUGCCAUC <u>AAAAAC</u> -Cy5
M40 (mut2)	AGCCCGAACACUACACACUACC U CGGGCAGCCAUGCCAUC <u>AAAAAC</u> -Cy5
F40 (Wt)	AGCACGAACCCAUAUUCGUCCUUCAAUUAUGGGUACUAU <u>AAAAAC</u> -Cy5

These are the first 40 bases of each viral (or mutated viral) 5'UTR with an additional 4 As at the 3' end to discourage 3' secondary structure which may impede ligation of the pCp-Cy5 label. This gives a final RNA probe length of 45 bases (including the label). Templates for T7-IVT were generated by PCR by using overlapping primers to give a DNA product consisting of the above sequences (ending AAAA) fused to a T7 promoter sequence at the 5' end (GTAATACGACTCACTATT). The optimal T7 promoter (TAATACGACTCACTATAGGG) includes the first three transcribed bases (underlined) and therefore cannot be used when a different 5' sequence is needed. Therefore, a class II T7 promoter (TAATACGACTCACTATA) was used which can initiate transcripts with an A, albeit at lower levels of activity than the optimal T7 promoter³¹³. Despite this, the class II promoter produced adequate amounts of RNA for this study, although the transcripts starting AGC were noticeably less-well transcribed than those starting AGG (data not shown). An attempt was made to produce the previously published VEEV RNAs²⁰⁸ which start ATG and ATA, but negligible amounts of RNA were produced, suggesting that positions +2 and +3 of the class II promoter are important for its activity (data not shown).

An unexpected issue was encountered during in vitro transcription of these RNA probes. When separating the products of the T7-IVT reaction on a high-percentage denaturing polyacrylamide gel, instead of the single expected 44-mer product, multiple bands were seen, especially for NP and F (figure 4.9A). Adjusting the concentration of Mg²⁺ in the reaction had no effect on this 'laddering' (data not shown). A search of the literature provided examples of how this laddering effect can be produced as a result of 'self-priming'^{314,315}. This requires the 3' end of the transcript to fold and bind to an upstream sequence on the same molecule or a complementary sequence on a different molecule. This short dsRNA is weakly recognised by T7 RNA polymerase (even in the absence of a promoter) and results in additional unwanted extensions to the 3' end of the transcript. It is not clear why this occurred with these PIV5 5'UTR sequences but not with the

transcripts of similar length used in published studies¹⁸⁰, nor why F produces both shorter and longer products. The only solution found was to introduce a Hepatitis D ribozyme (HDR) sequence 3' of the RNA probe sequences listed above. The HDR is designed to cleave following the AAAA sequence and thus generate the desired 3' end of each transcript without the unwanted extensions. This approach was successful and produced homogenous products of the expected length which could be purified from the gel and used for downstream enzymatic modifications. The large, slow-migrating products shown in figure 4.9A are those of the cleaved 3' HDR and the full-length uncleaved transcript; the differing cleavage efficiency (for example, compare P and M) is due to sequences upstream of the HDR in the M 5'UTR affecting folding of the ribozyme (structure predictions not shown). An unfortunate consequence of using the HDR is that cleavage produces a 3' end bearing a 2,3-cyclic phosphate (cP) which must be dephosphorylated (using T4 PNK) to leave the 3'OH which is the required substrate for T4 ssRNA ligase.

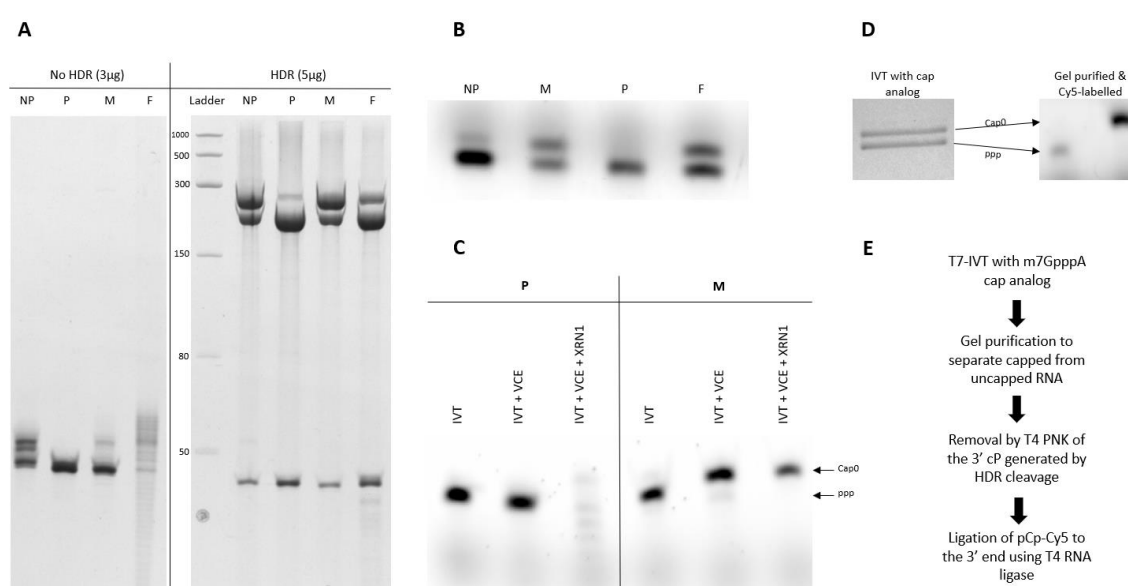


Figure 4.9 - Generation of capped RNA probes for EMSA. A) T7 IVT reactions using templates either without or with an HDR to generate the intended 3' end. The indicated amount of total RNA from the IVT reaction run on a denaturing gel and stained with methylene blue. A ssRNA ladder is shown, labelled in nucleotides. **B)** Gel-purified 44-mer HDR cleavage products were capped with Vaccinia capping enzyme following the recommended protocol, then RNA was 3'-labelled with Cy5 and separated on a denaturing gel. **C)** Gel-purified 44-mer HDR cleavage products were either untreated (IVT), treated for 16h with Vaccinia capping enzyme (VCE), or treated with VCE followed by RPP and XRN1, and run on a denaturing gel to separate capped (Cap0) from uncapped (ppp) RNA. **D)** The same template as before (HDR cut-off not shown) was used in an IVT reaction with a 1:4 ratio of ATP to m7GpppA cap analog and run on a denaturing gel to separate capped (Cap0) from uncapped (ppp) RNA. Each product was separately gel purified and labelled with Cy5. The final products were run on a denaturing gel side-by-side to confirm separation of ppp and Cap0 RNA. All gels are 7.5M urea, 14% polyacrylamide. **E)** A schematic showing the final protocol used to generate labelled RNA probes for EMSAs.

A second issue was encountered when attempting to cap the RNA purified from the gel shown in figure 4.9A. Vaccinia capping enzyme (VCE) was used according to manufacturer instructions and, following Cy5-labelling, the capped RNA probes were run on a high-percentage polyacrylamide gel. As shown in figure 4.9B, multiple products were present for M, F, and NP, while P produced a single, faster-migrating product. The slower-migrating product is capped RNA and the faster-migrating product is uncapped (the addition of m7G provides a sufficient size difference for the two to be separated by electrophoresis). The apparent inability of VCE to cap the P 5'UTR, and to a lesser extent the NP 5'UTR, correlates with the predicted secondary structure of these RNAs (figure 4.2) suggesting that VCE requires an overhang to bind the 5' end of RNA, much like IFIT1. One study has determined the length of unpaired 5' overhang required for VCE activity and found that 2 bases are sufficient while no overhang completely inhibited activity³¹⁶. The effect of a 1-base overhang was not tested but the results seen with P suggest that VCE requires a minimum of a 2-base overhang for capping activity.

XRN1 binds and degrades 5' monophosphorylated RNA. RNA 5' polyphosphatase (RPP) recognises ppp-RNA – but not capped RNA – and leaves a 5' monophosphate. Thus, sequential treatment of a mixed population of capped and uncapped transcripts with RPP and XRN1 enriches capped RNA at the expense of uncapped. This approach has been used to overcome incomplete capping by VCE and obtain a sample of completely capped RNA^{317,318} and so was attempted with P40. The VCE reaction time was extended from 1h to 16h at 37°C followed by short incubations at 42°C and 50°C in the hope that the RNA secondary structure would be more affected by the increased temperature than the VCE. The RNA was also heat-denatured and fast-cooled on ice prior to addition of the reaction components, again to discourage RNA secondary structure formation. Despite these measures, the P40 RNA remained completely resistant to capping by VCE, as shown by a single band being present both before (IVT) and after (IVT+VCE) capping (figure 4.9C). Subsequent treatment with RPP and XRN1 resulted in total RNA degradation, showing that these enzymes do not require a 5' overhang longer than 1 base. However, the lack of capped P40 RNA present in the sample meant there was insufficient RNA left following XRN1 treatment for this to be a viable approach to obtain capped P40 RNA. In contrast, the M40 RNA reached near-total capping following the extended VCE reaction and the small amount of uncapped RNA was successfully removed by XRN1.

The only remaining option to obtain capped P40 RNA was to circumvent the issue of 5' structure inhibiting enzymatic modification by using a cap analog in the T7-IVT reaction. Reducing the concentration of ATP and using a high concentration of the commercially available m7GpppA cap analog (in a 1:4 ratio) favours the incorporation of m7GpppA at the first position of the transcript rather than A. This should result in 80% of transcripts being capped and 20% of

transcripts being uncapped. Although this mixed population could be resolved by RPP/XRN1 treatment as in figure 4.9C, the use of the HDR means that the products of the T7-IVT reaction include the HDR cut-off which will have a 5'OH structure not recognised by XRN1. To remove this, the 44mer RNA must be gel purified, so the mixed capped/uncapped population was separated on a denaturing gel and then purified to obtain a completely capped sample. As shown in figure 4.9D, there was an approximately equal split between capped and uncapped product despite the 4:1 ratio of cap analog to ATP, suggesting that T7 RNA polymerase favours ATP over the analog as a substrate. Despite this, an adequate amount of capped RNA was obtained for labelling and the purity of this capped RNA was checked by running on a denaturing gel next to the uncapped RNA processed in parallel (figure 4.9D, right).

This approach was then used to generate all of the 7 RNA probes listed at the start of this section. For each probe, the uncapped and capped products from the cap analog T7-IVT reaction were cut out of the gel and labelled in parallel so that their purity could be demonstrated. As shown in figure 4.10, the cap0 probes for P40, M40, P40(mut1), and M40(mut1) were completely separated from uncapped product. The other cap0 probes contained a small amount of uncapped RNA that was unable to be separated fully when cutting out of the gel. However, the amount of contamination is a very small proportion of the total RNA in each sample and is perhaps comparable to what would result from incomplete capping of unstructured RNA by VCE, as is standard in publications on IFIT1. Note that the Cy5 labelling efficiency was found to vary widely between reactions, so that despite an equal amount (1 pmol) of RNA being loaded in each gel lane, the fluorescent intensities of each RNA probe are not equal. M40(mut2) was poorly labelled (not shown) and therefore not tested.

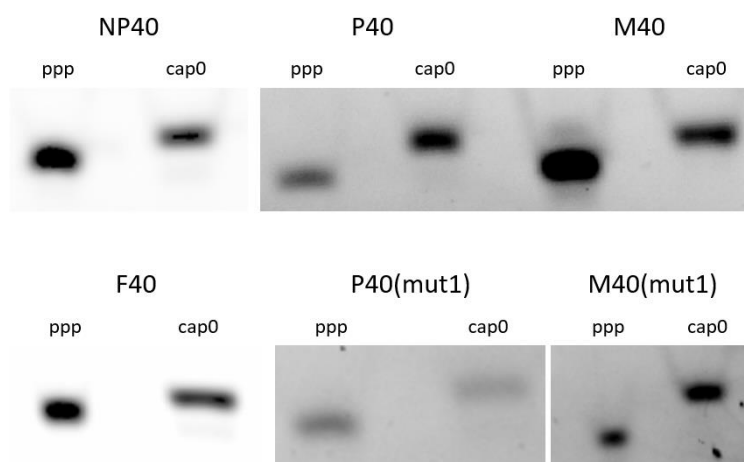


Figure 4.10 - Confirmation of correct cap structures on RNA probes. 1 pmol of the indicated Cy5-labelled RNA probes was run on a high-percentage denaturing polyacrylamide gel (14%, 7.5M urea) and imaged using a range of exposures suitable for the efficiency with which each probe had been labelled.

This was a very laborious process but was the only way to generate completely-capped RNA when the 5' end is structured. For most applications, 5' RNA structure can simply be avoided when designing RNA for transcription. However, even unstructured RNA may be incompletely capped by VCE, as shown by the incomplete capping of M in figure 4.9B and even after extended incubation in figure 4.9C. Many studies of IFIT1 and capped RNA have relied on the efficiency of VCE being high rather than ensuring complete capping by either gel purification or chemical synthesis. This is a particular issue for the previous study of the impact of secondary structure, which included EMSAs of IFIT1 binding to VEEV RNA with a 1-base overhang²⁰⁸. Hyde *et al.* performed capping with VCE but neither confirmed successful capping nor made an attempt to separate capped from uncapped RNA. The presence of uncapped RNA would decrease the affinity of IFIT1 relative to a sample of completely cap0 RNA. However, the presence of secondary structure would affect both capping and IFIT1-binding, so the conclusion is likely valid, if not the dissociation constants obtained from the filter-binding assay.

4.3.2 Purification of recombinant hIFIT1 and mIFIT1

Recombinant hIFIT1 and mIFIT1 were purified in a similar manner to the previously published methodology^{178,253}. The cap-binding mutant hIFIT1(R187H) was also purified to confirm that any RNA binding by hIFIT1 is dependent upon its binding to the 5' end of RNA. Briefly, plasmids expressing His-tagged IFIT1 under control of an IPTG-inducible promoter were transformed into *E. coli* strain BL21(DE3). Following lysis, His-tagged protein was separated using Ni-NTA magnetic beads and the final eluted protein analysed by SDS-PAGE (figure 4.11A).

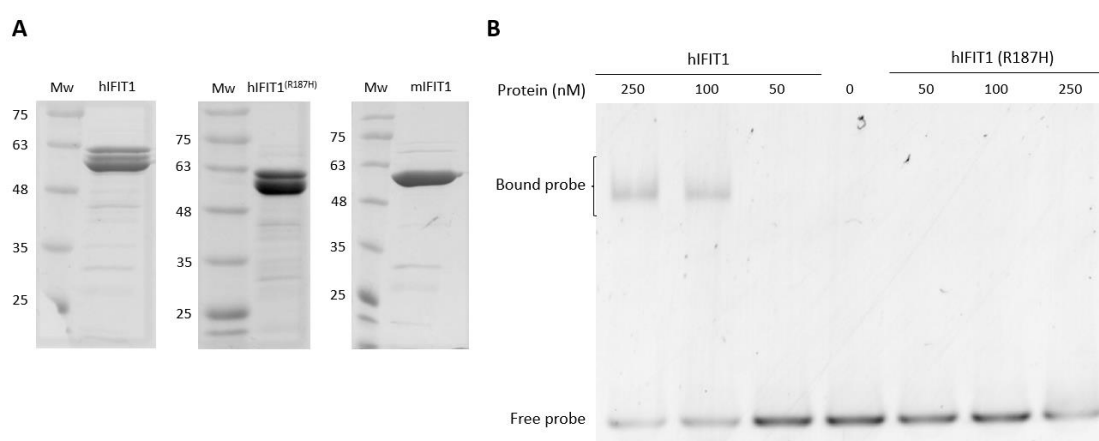


Figure 4.11 – Purification of recombinant IFIT proteins. A) Coomassie-stained SDS-PAGE gel showing the final eluted recombinant hIFIT1, hIFIT1(R187H), and mIFIT1 proteins. Molecular weight markers are shown in the left-hand lanes, labelled in kDa. **B)** EMSA for hIFIT1 and hIFIT1(R187H) binding to the unstructured cap0 M40 probe. 40nM of Cy5-labelled probe was allowed to bind at the indicated concentration of either hIFIT1 (left) or hIFIT1(R187H) (right) protein. Complexes were run on a native polyacrylamide gel and imaged using a ChemiDoc MP system. The running position of the unbound free probe and the shifted bound complexes are indicated.

hIFIT1 migrated at approximately 56 kDa as expected, but co-eluted with numerous smaller protein species at low concentrations, and two larger products. mIFIT1 also migrated at the expected size, but was of higher purity than hIFIT1 and co-eluted with fewer other products. The lack of purity of these protein preparations, in particular hIFIT1, whether due to non-specific binding to the Ni-NTA beads or due to cleavage of the protein, precludes a direct comparison between the activity of hIFIT1 and mIFIT1 and will affect the concentration at which they are found to bind RNA. However, this chapter is primarily focussed on the comparison between RNA species rather than IFIT proteins. The impact of cap-proximal predicted RNA secondary structure on binding shown in the next section suggests that the purified hIFIT1 and mIFIT1 proteins are both functional and are able to bind the 5' end of RNA as expected. The loss of RNA-binding caused by the R187H change in hIFIT1 (figure 4.11B) confirms that the interaction of hIFIT1 with the labelled probe is specific to the 5' end of the RNA.

4.3.3 Comparison of PIV5 5'UTRs by EMSA with IFIT proteins

As the RNAs predicted to have the strongest and weakest 5' structure, the P40 and M40 RNAs were chosen for an initial titration of both hIFIT1 and mIFIT1. While keeping the RNA concentration constant at 40nM, the concentration of protein was varied between 0nM and 1500nM to cover a range of concentrations used in published IFIT1 in vitro experiments¹⁸⁰. Prior to addition of the protein, RNA was heat-refolded by heating to 70°C and being allowed to cool for 15 minutes before adding the binding buffer, which contains MgCl₂ (final concentration 5mM) and KCl (final concentration 10mM), and leaving for a further 15 minutes at room temperature to allow RNA folding in the presence of positively charged ions. The protein was then added and allowed to bind for 30 minutes before running the reaction on a native polyacrylamide gel. This protocol should allow for any RNA structure to form and affect IFIT1-binding.

Figure 4.12 shows the binding of P40 and M40 to hIFIT1 (figure 4.12A) and mIFIT1 (figure 4.12B). Both proteins have much higher affinity for M40 than for P40, with shifted complexes of M40 being visible at 50-100nM hIFIT1 and as low as 25nM for mIFIT1. In contrast, shifting of P40 required much higher concentrations: 500nM for hIFIT1 and 100nM for mIFIT1. Also notable was that even at very high concentrations (1500nM, roughly the concentration of hIFIT1 reached in cells at the peak of the IFN-induced antiviral state²⁵³), P40 was never fully-shifted; a small amount of free-probe was always still visible. Full-shifting of M40 was achieved at about 500nM

hIFIT1 and 250nM mIFIT1, much lower concentrations than those likely to be present in cells exposed to IFN. Another noticeable difference between P40 and M40 was that complexes formed with M40 at low protein concentrations were of different electrophoretic mobility than those with either P40 or M40 at higher concentrations. For example, the complex formed at 25-100nM mIFIT1 with M40 is not seen at all with P40. This is likely to be a result of the protein oligomerising at higher concentrations^{162,163,179}. The smaller complex could represent either monomeric or dimeric mIFIT1, while the larger complex could represent tetrameric or even higher order oligomers. This may also explain why at high concentrations of protein, the RNA:protein complex appears to get 'stuck' in the gel well.

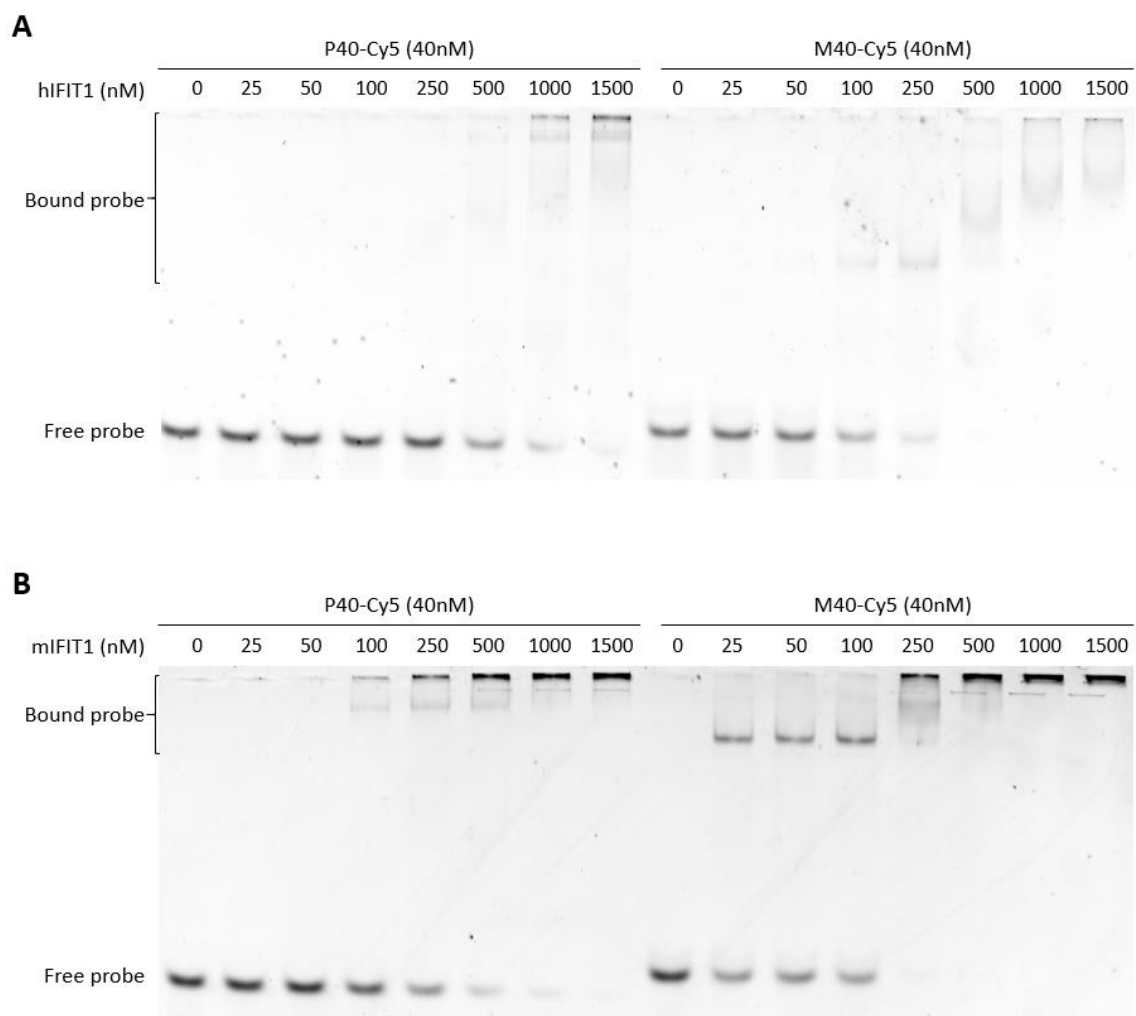


Figure 4.12 - EMSA for hIFIT1 and mIFIT1 binding to P40 and M40. 40nM of Cy5-labelled P40 or M40 cap0 RNA probe was heat-refolded and then allowed to bind at the indicated concentration of either hIFIT1 (A) or mIFIT1 (B) protein. Complexes were run on a native polyacrylamide gel and imaged using a ChemiDoc MP system. The running position of the unbound free probe and the shifted bound complexes are indicated.

P40 and M40 clearly differ greatly in their affinity for IFIT1. Having established the range of protein concentrations that emphasise this difference, the NP40 and F40 RNAs were also tested in the same manner. The Cy5 labelling reaction varies in efficiency and the NP40 and F40 probes were labelled to a greater extent than the P40 and M40 probes, which were generated separately. Therefore, the NP40 and F40 sections of the gels in figure 4.13 are shown at a different exposure and any apparent differences in the amount of each probe used reflects the varying levels of Cy5 ligation rather than a difference in RNA concentration, which is maintained at a constant 40nM across all probes. As before, P40 displayed a much lower affinity for both hIFIT1 (figure 4.13A) and mIFIT1 (figure 4.13B) than M40. NP40 was found to have a higher affinity for both proteins than P40, but a lower affinity than M40; a greater ratio of shifted to unshifted probe was seen for NP40 relative to P40 at 500nM hIFIT1 and 250nM mIFIT1. However, free NP40 probe was still clearly visible at 500nM of both proteins, showing that it has a much lower affinity for IFIT1 than M40, which was either fully shifted (mIFIT1) or mostly shifted (hIFIT1) at this concentration. In contrast, F40 displayed a similar affinity for IFIT1 as M40, being partially shifted at low concentrations and fully shifted at 500nM of both proteins.

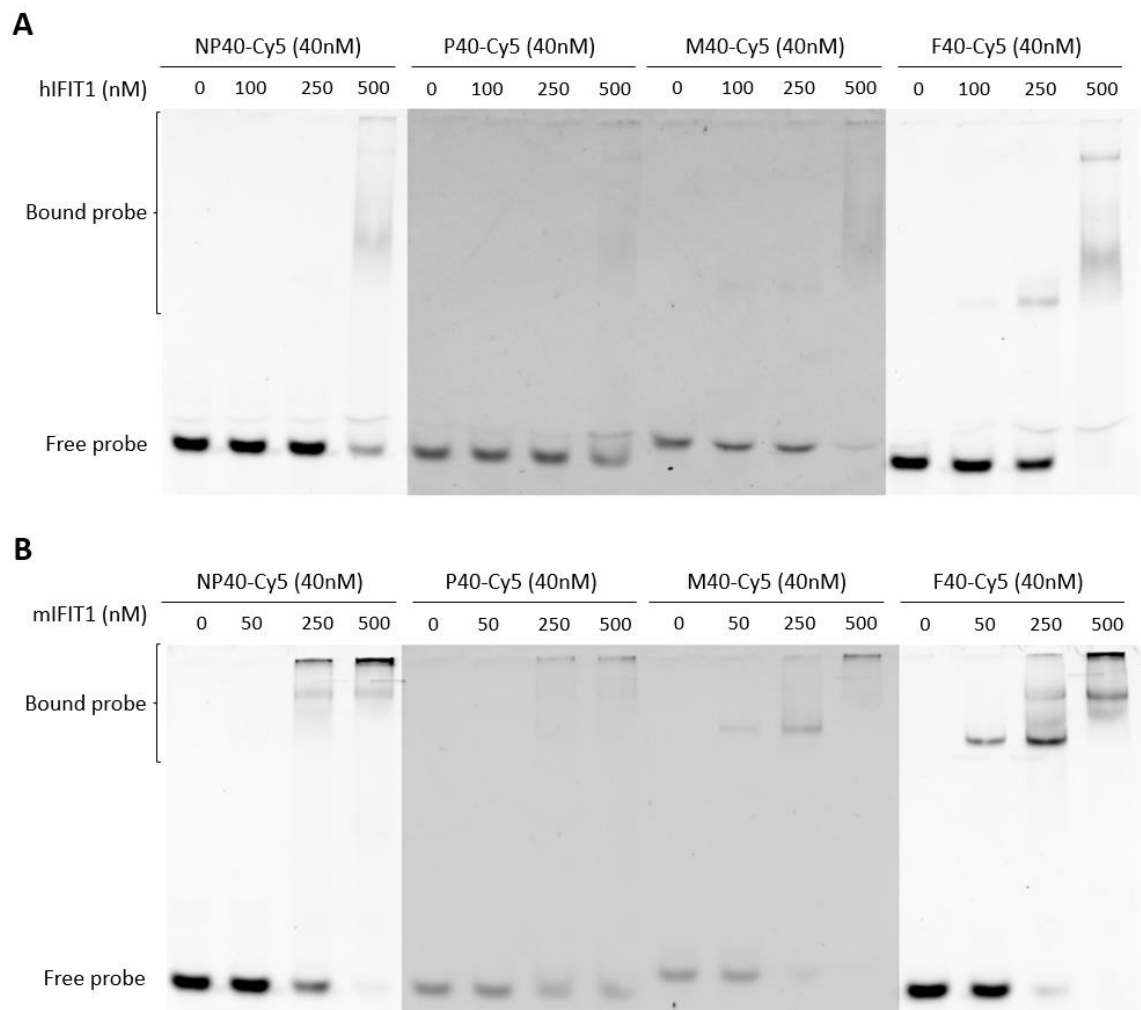


*Figure 4.13 - EMSA for hIFIT1 and mIFIT1 binding to NP40, P40, M40 and F40. 40nM of Cy5-labelled cap0 RNA probe was heat-refolded and then allowed to bind at the indicated concentration of either hIFIT1 (**A**) or mIFIT1 (**B**) protein. Complexes were run on a native polyacrylamide gel and imaged using a ChemiDoc MP system. The running position of the unbound free probe and the shifted bound complexes are indicated. Due to the varying efficiency with which each probe was labelled, different exposure times are shown for different sections of each gel. Note that the ratio of free to bound probe for each RNA should be compared rather than the intensity of free probe.*

The above results correlate with the predicted secondary structure of each RNA in that those with strong 5' structure (NP40 and P40) require a much higher concentration of IFIT1 to be shifted than those with no predicted 5' structure (M40 and F40). However, it cannot be excluded that the 5' sequence is responsible for these differences rather than RNA secondary structure. For example, both NP40 and P40 begin AGG while M40 and F40 begin AGC; a pyrimidine at position 3 could in theory be more easily accommodated in the IFIT1 RNA-binding pocket than a purine (although IFIT1 is predicted to interact with the RNA backbone rather than the bases¹⁸⁰). To confirm that secondary structure is the determinant of differing IFIT1 binding affinity, RNAs with downstream mutations were tested. As previously described, these changes either introduce (M40(mut1)) or disrupt (P40(mut1)) the predicted 5' structure while maintaining the same terminal sequence.

Firstly, P40 was tested at a range of concentrations (0-500nM hIFIT1 and 0-250nM mIFIT1) previously shown to shift the unstructured M40 RNA (figure 4.14). As before, the P40 RNA was highly resistant to binding by both hIFIT1 and mIFIT1 and no shifted complexes were visible at these concentrations. In contrast, for P40(mut1) shifted complexes were visible at 250nM hIFIT1 and 100nM mIFIT1, showing that IFIT1 has much higher affinity for this mutated RNA than the unedited P40. P40(mut1) was not shifted as much as M40 was at low concentrations (figure 4.12, not compared directly) which suggests that a 4-base 5' overhang (the structure predicted for P40(mut1), see figure 4.4) is not sufficient for maximal binding of IFIT1, as implied by toeprinting analysis of hIFIT1 showing a potential need for a 7-base overhang¹⁷⁹. However, this result with P40(mut1) shows that the strong predicted 5' structure of P40 is likely responsible for its relative resistance to IFIT1-binding.

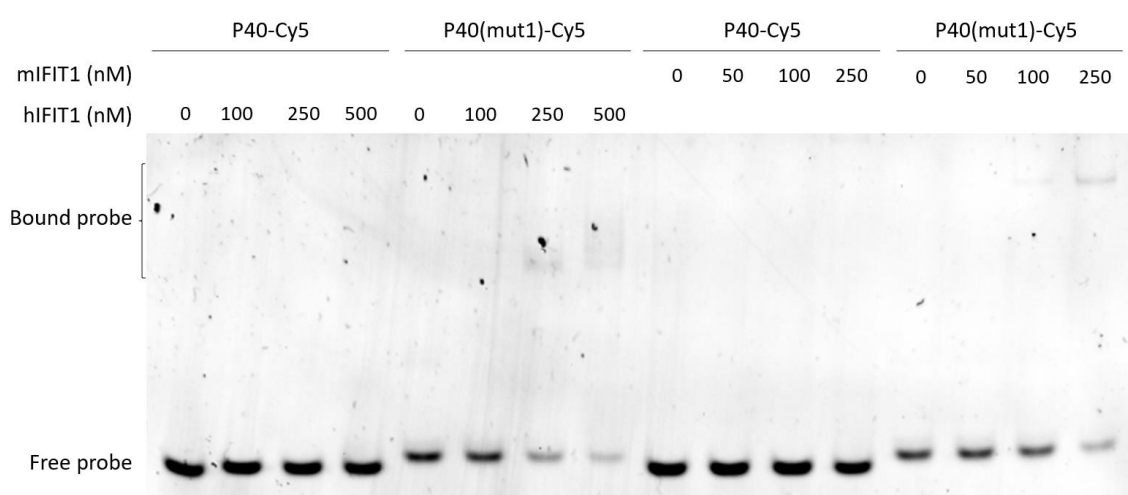


Figure 4.14 - EMSA for hIFIT1 and mIFIT1 binding to P40 and P40(mut1). 40nM of Cy5-labelled P40 or P40(mut1) cap0 RNA probe was heat-refolded and then allowed to bind at the indicated concentration of either hIFIT1 or mIFIT1 protein. Complexes were run on a native polyacrylamide gel and imaged using a ChemiDoc MP system. The running position of the unbound free probe and the shifted bound complexes are indicated. The differing intensities between probes is due to varying Cy5 labelling efficiencies; the RNA concentration is the same.

Next, the M40(mut1) UTR was tested in the same manner (figure 4.15). The introduction of predicted structure in M40(mut1) rendered it resistant to binding by both hIFIT1 and mIFIT1 at low concentrations, in contrast to M40 which is shifted by just 100nM hIFIT1 and 25nM mIFIT1. While M40 was completely shifted by 500nM hIFIT1 or 250nM mIFIT1, unbound M40(mut1) probe was still visible even at the highest protein concentrations used here. The reduced affinity for IFIT1 of the altered M UTR shows that in the absence of cap1 structure, 5' RNA secondary structure determines the sensitivity of RNA sequences to binding by IFIT1. The presence of unbound M40(mut1) probe even at high concentrations of IFIT1 suggests that the introduction

of 5' structure to viral mRNAs would be able to restore some level of translation during the IFN-induced antiviral state.

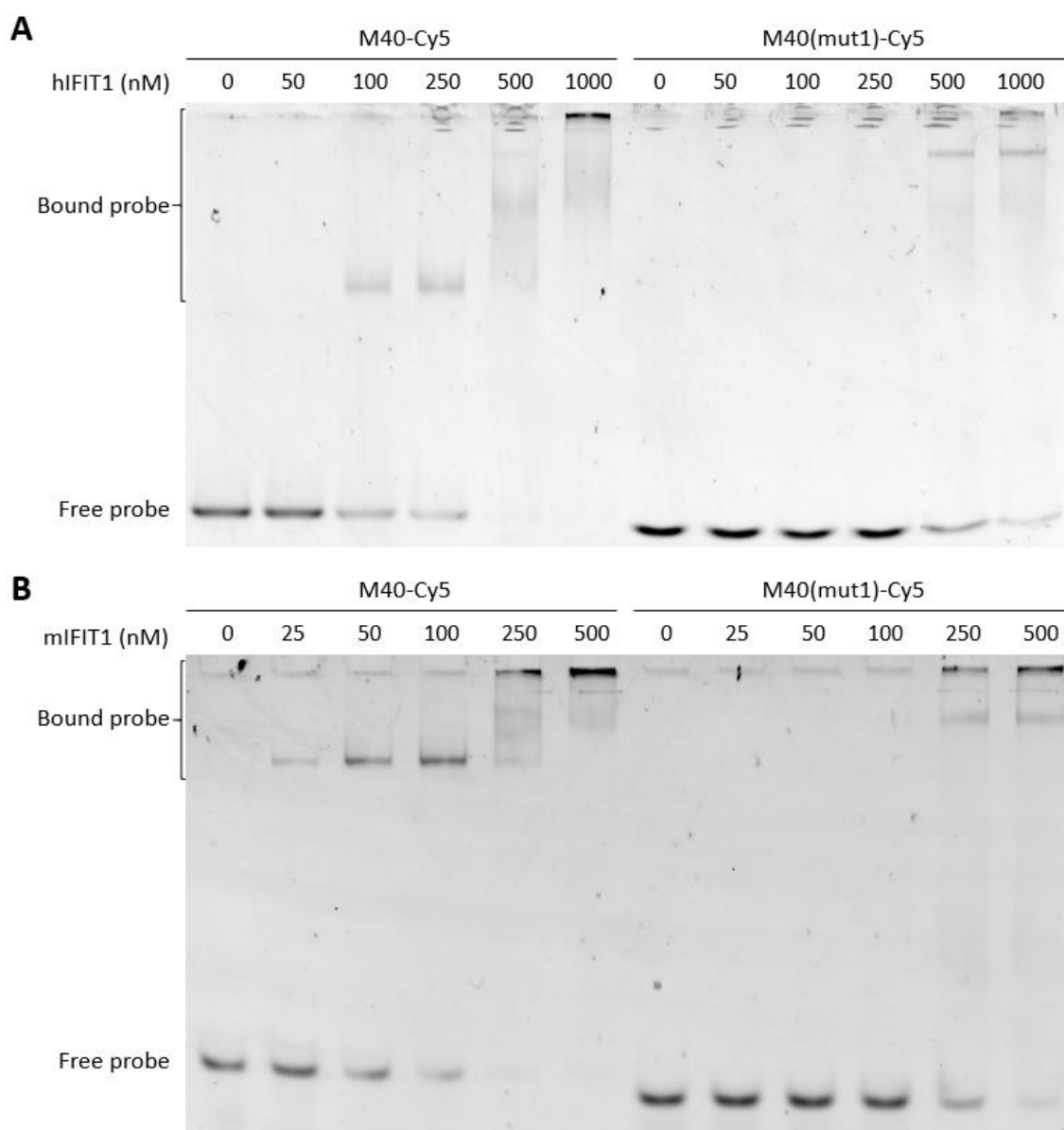


Figure 4.15 - EMSA for hIFIT1 and mIFIT1 binding to M40 and M40(mut1). 40nM of Cy5-labelled M40 or M40(mut1) cap0 RNA probe was heat-refolded and then allowed to bind at the indicated concentration of hIFIT1 (A) or mIFIT1 (B) protein. Complexes were run on a native polyacrylamide gel and imaged using a ChemiDoc MP system. The running position of the unbound free probe and the shifted bound complexes are indicated. The differing intensities between probes is due to varying Cy5 labelling efficiencies; the RNA concentration is the same.

4.3.4 Comparison of the M and M(mut1) 5'UTRs by in vitro translation in the presence of IFIT1

Although the EMSA results presented above are strong evidence for secondary structure determining the differing sensitivity of PIV5 5'UTRs to IFIT1, a weakness of the EMSA is that it is

not able to distinguish between biologically relevant and irrelevant RNA:protein interactions. In the context of IFIT1, this could be either interaction with RNA at a location other than the 5' cap, or an interaction shown by EMSA but that is not strong enough to outcompete translation factors such as eIF4E in the cytoplasm. These possibilities can be excluded by using in vitro translation, which has been used previously to study IFIT proteins^{180,247,253} and can link the affinity of IFIT1 for RNA shown by EMSA to its function in cells, i.e. outcompeting translation factors to prevent translation of cap0 mRNA.

For this experiment, the M40, M40(mut1), and M40(mut2) sequences were selected. The template for T7-IVT was generated by amplifying by PCR the relevant region of the BiMG-M40 plasmid used in the previous chapter with a forward primer containing the T7 promoter and binding to the M transcription start site, and a reverse primer binding the L 3'UTR gene end site. This produces a template consisting of the first 40 bases of the M'UTR (M40, including the upstream start codon); a short AACC non-viral sequence to maintain the reading frame from the start codon upstream of the Fluc CDS; the Fluc CDS; and the L 3'UTR. The predicted structure of M40-Fluc, M40(mut1)-Fluc, and M40(mut2)-Fluc is the same at the 5' end as the predicted structure of the 40-mer sequences shown previously (figure 4.16A).

These M40-Fluc reporter transcripts need to be capped at the 5' end both so that they are translated efficiently in the rabbit reticulocyte lysate (RRL) in vitro translation system and so that the cap-binding of IFIT1 can be investigated. For most applications, mRNA generated by T7-IVT is capped post-transcriptionally using VCE. However, as established in the previous section, VCE is severely affected by 5' structure leaving an overhang of 1 base or less, meaning that it is not possible to use VCE to cap the M40-Fluc mutant reporter transcripts and guarantee equal capping efficiency between them. To avoid potential bias introduced by enzymatic modifications of the 5' ends, the T7-IVT reaction was instead performed with the m7GpppA cap analog in a 4:1 ratio with ATP. It is not possible to separate uncapped from capped long transcripts on a gel, so a mixed population of ppp and cap0 RNA was used for in vitro translation. The previous results with the cap analog show that about 50% of RNA is capped (figure 4.9D), but capping provides a strong increase in translatability so Fluc signal seen in the RRL system will predominantly be from capped RNA rather than uncapped. Removal of uncapped transcripts could be performed by sequential treatment with RPP and XRN1, as demonstrated in the previous section (figure 4.9D), but without a way to verify the cap status of the final product, it is not possible to exclude the possibility that uncapped structured transcript (e.g. M40(mut1)) would be less well-degraded than the unstructured M40-Fluc. The final in vitro transcribed mRNAs lack a true polyA tail, having only the 6 adenines encoded at the end of the transcript in the viral genome. Although required for efficient translation in cells, polyadenylation is not required for translation

in nuclease-treated RRL³¹⁹. An initial check of the translatability of the mutant 5'UTRs was made relative to the unchanged M40 5'UTR (figure 4.16B). In contrast to the results with the BiMGs which weakly suggested a negative impact of secondary structure on translation, no such effect was found here.

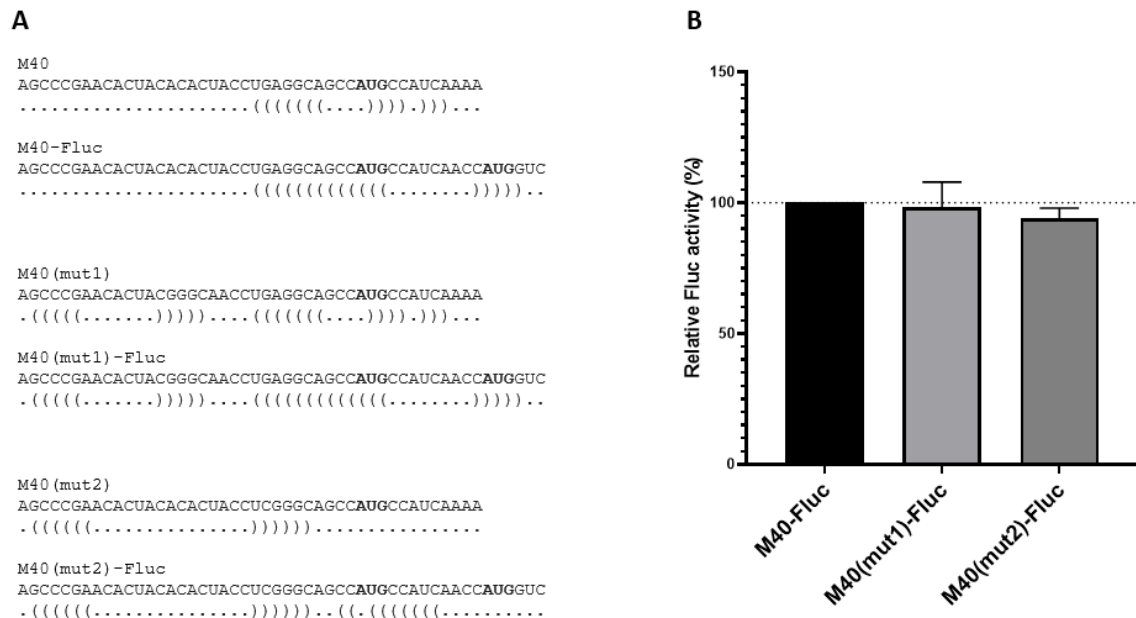


Figure 4.16 - RNA structure predictions for in vitro transcribed M40-Fluc mRNA. A) The entire sequence of the M40-Fluc transcript (and its mutated versions) were submitted to the RNAfold server, as before. The predicted structures at the 5' end of the transcripts are shown in dot-bracket notation and compared to the 40mer predictions previously shown in this chapter. Start codons, including the one in the M40 sequence upstream of the Fluc start codon, are shown in bold. **B)** Translation activity of M40(mut1)-Fluc and M40(mut2)-Fluc in the RRL system relative to M40-Fluc. Error bars are SEM and data are an average of three independent replicates.

The in vitro transcribed M40-Fluc mRNAs were then translated in the RRL system at varying concentrations of hIFIT1 and mIFIT1 (figure 4.17). Sensitivity to mIFIT1 was found to correlate with a lack of predicted 5' structures: M40-Fluc was more sensitive than M40(mut1)-Fluc and M40(mut2)-Fluc at all concentrations of protein. M40(mut1)-Fluc was found to be slightly less sensitive than M40(mut2)-Fluc, and at low concentrations (100nM and 250nM) of mIFIT1, translation of M40(mut1)-Fluc was actually increased. This effect has been seen previously during in vitro translation experiments at low concentrations of IFIT1^{180,253}.

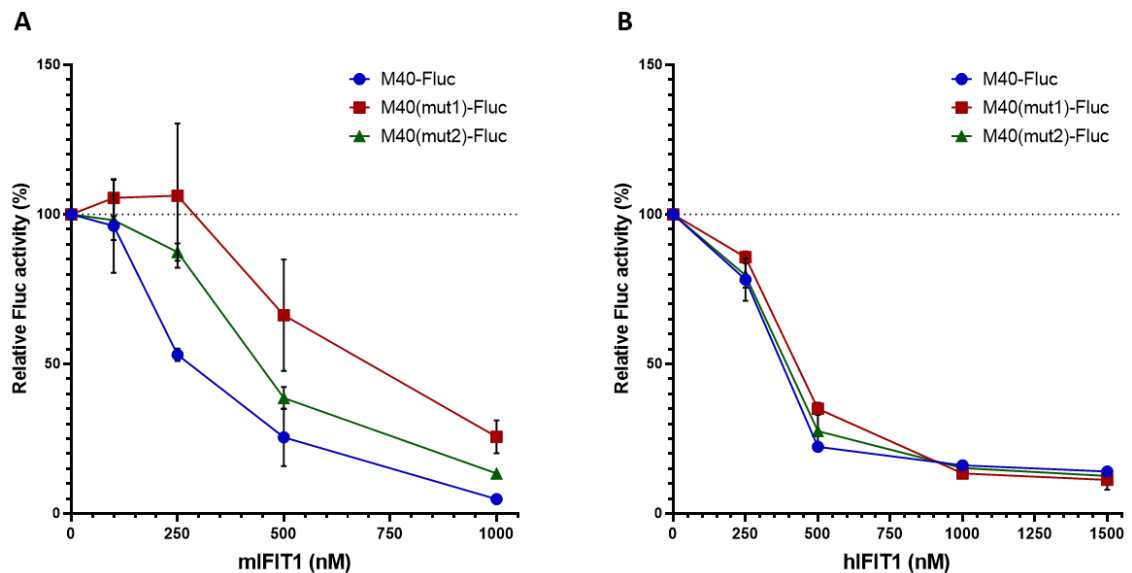


Figure 4.17 - Inhibition of *in vitro* translation of the M UTR by hIFIT1 and mIFIT1. M40-Fluc, M40(mut1)-Fluc, and M40(mut2)-Fluc were *in vitro* transcribed in the presence of m7GpppA cap analog and then translated in the RRL system at a concentration of 10nM in the presence of the indicated concentration of either mIFIT1 (A) or hIFIT1 (B) protein. Error bars are SEM and the data are an average of three (A) or two (B) independent replicates.

In contrast to mIFIT1, inhibition of reporter mRNA by hIFIT1 was not found to differ between 5'UTRs, except for a very small effect at 500nM (figure 4.17B). It is not clear why inhibition of translation by mIFIT1 would be affected by mRNA 5' structure but inhibition by hIFIT1 would not be, especially since both proteins were shown to be affected by the increased 5' structure of M40(mut1) in the EMSAs in the previous section (figure 4.15). hIFIT1 could be having a non-cap-specific effect on translation, but cap methylation by CMTR1 has previously been shown to reduce inhibition by hIFIT1 in the RRL system¹⁵⁰, suggesting that its activity is indeed dependent upon binding to mRNA caps. Nevertheless, the results seen with mIFIT1 show that the altered M 5'UTRs can reduce the inhibitory effect of IFIT1 on translation.

4.4 The IFIT1-sensitivity of PIV5 with mutated 5'UTRs

Having established that the 5' structure of PIV5 5'UTRs varies and affects the binding of IFIT1, the effect of downstream mutations in the 5'UTRs of PIV5 was investigated in the context of the virus itself. This is important for several reasons: 1) it shows whether the differences in 5'UTR structure can affect IFIT1-binding when the protein is present at high levels during the IFN-induced antiviral state, 2) sequences downstream of 40 bases (the length investigated in the previous sections) in the viral mRNAs could cause different structures to form, and 3) it allows for demonstration of the consequence of differing transcript sensitivity on viral fitness. The four

mutations previously described – P(mut1), M(mut1), M(mut2), and F(mut1) – were introduced into the virus and the effect on viral protein synthesis and fitness investigated by western blot and growth curves.

Because the MTase activity of the VSV L protein displays sequence-specificity¹⁹⁸, we had originally hypothesised that the 5'UTRs of PIV5 genes may be differentially methylated by L due to their varying sequences. An attempt to alter the 5'UTRs of PIV5 by making changes at position 3 was unsuccessful because these viruses were not able to be rescued (Rick Randall, personal communication), possibly due to disruption of the authentic gene start signals. In hindsight, this approach was not ideal because of the evidence since acquired that PIV5 transcripts are likely all cap0. Making changes downstream of the 5' end of the UTR is preferable because it avoids disruption of the gene start site and allows for confirmation of the role of secondary structure while excluding any possible impact of the variation as position 3 in PIV5 5'UTRs.

4.4.1 Confirmation of virus rescue

PIV5/F157/mCherry was selected for these experiments. Relative to the PIV5/W3A reference strain, this virus expresses mCherry from an inserted gene between HN and L as well as a single amino acid change in P/V which results in increased RNA polymerase activity^{38,224}. The increased rate of RNA synthesis of an F157 virus is advantageous because it prevents the shutoff of viral transcription seen at late timepoints post-infection with W3A²²⁴ which would both affect detection of protein levels and also make any differences in viral growth caused by IFIT1 less obvious. PIV5 strains encoding F157 are also more potent inducers of IFN (Matthew Pickin, data unpublished), so the F157 virus is a more relevant model for studying the effect of an ISG. The mCherry reporter gene makes viral rescue and titration much simpler, and also provides an easily-detectable readout of viral protein synthesis. As previously mentioned in chapter 3, the incorporation of mCherry into the viral genome reduces virus-induced cytopathic effect (CPE) (Rick Randall, personal communication), but this disadvantage is overcome by detecting plaques by mCherry expression rather than relying on CPE and crystal violet staining of fixed cells.

The S157F change and the insertion of mCherry were made by Steve Goodbourn to the full-length cDNA PIV5 clone plasmid pBH276²¹³, which contains the W3A viral genome sequence under control of a T7 promoter. The new plasmids with the altered 5'UTRs were then used to rescue virus in BSRT7 cells, as previously described²¹³. For brevity, the viruses are named F157/P1, F157/M1, F157/M2, and F157/F1, while the virus with unchanged UTRs is named PIV5/F157/mCherry or simply F157. The predicted secondary structure of the mutated viral

mRNAs was confirmed by submitting them to the CoFold program, as before (figure 4.18). Also shown is the mCherry UTR which is predicted to form weak 5' secondary structure and therefore would be expected to be less sensitive to IFIT1 than M, but more sensitive than P and NP.

```
mCherry
AGGCCCGGACGGGUUAGCAACAAGCGCGGCCGCAAUGGUGAGCAAGGGCGAGGAGGAUA
..(((((((..(((((..(((..))))..))..))..))..))..))..))..(((((((..

NP
AGGUCCGGAACCUAUGGCCUUCGUGACCGACCUAGAGUCAGAGUAGUUAUAAGGACCU
..(((((((..(((((..(((..))))..))..))..))..))..))..(((((((..(((..

P
AGGCCCGGACGGGUUAGCAACAAGCGACUGCCGGUGCCAACAGCGCAAUCCACAAUCUAC
..(((((((..(((((..(((..))))..))..))..))..))..))..(((((((..

P (mut1)
AGGCCCGGACGGGUUAGCAACAAGCGACUGCCGGUAAAACAGCGCAAUCCACAAUCUAC
....(((((((..(((((..(((..))))..))..))..))..))..))..(((((((..

M
AGCCCGAACACUACACACUACCUGAGGCAGCCAUGCCAUCCAUCAGCAUCCCGCAGACC
.....(((((((..(((((..(((..))))..))..))..))..))..(((((((..(((..

M (mut1)
AGCCCGAACACUACGGGCAACCUGAGGCAGCCAUGCCAUCCAUCAGCAUCCCGCAGACC
..(((((((..(((((..(((..))))..))..))..))..))..))..(((((((..(((..

M (mut2)
AGCCCGAACACUACACUACCUCGGGCAGCCAUGCCAUCCAUCAGCAUCCCGCAGACC
..(((((((..(((((..(((..))))..))..))..))..))..))..(((((((..(((..

F
AGCACGAACCCATATCGTCCTTCAAATCATGGGTACTATAATTCAATTTCTGGTGGTCTC
.....(((((((..(((((..(((..))))..))..))..))..))..(((((((..(((..

F (mut1)
AGCACGAACCCATATCGTCTGGCAAATCATGGGTACTATAATTCAATTTCTGGTGGTCTC
..(((((((..(((((..(((..))))..))..))..))..))..))..(((((((..(((..
```

Figure 4.18 - Co-transcriptional RNA secondary structure folding predictions for PIV5/F157/mCherry transcripts. The entire sequence of each PIV5 transcript was used for each prediction but only the first 60 bases are shown in dot-bracket notation. Highlighted in yellow are changes introduced to the UTRs to disrupt or form secondary structures. Also shown is the mCherry 5'UTR which consists of the first 26 bases of the P UTR followed by 9 inserted bases before the mCherry CDS. Predictions were made using the CoFold server with the default parameters (Turner, 1999).

All viruses were successfully rescued alongside the unaltered PIV5/F157/mCherry, as was clear by mCherry expression in both transfected BSRT7 cells and in Vero cells following transfer of cell supernatant (not shown). RNA was isolated from infected cells and, following RT-PCR, the mutated regions of the P, M and F 5'UTRs were sequenced (figure 4.19). The possibility of amplification from the transfected pBH276 plasmid was excluded by DNase treatment and a control PCR without RT (data not shown). The expected mutations were present and no minor peaks were detected in the trace, showing that no reversion had occurred during rescue and that the changes do not affect virus viability. When making stocks of these viruses, they all grew to a similar titre in Vero in cells (data not shown), again suggesting that these UTR changes have no major impact on viral fitness. Growing and plaquing the rescued viruses in Vero cells should

reduce any effect of differing IFIT1 sensitivity between the viruses because Vero cells lack type I IFN^{272,273}.

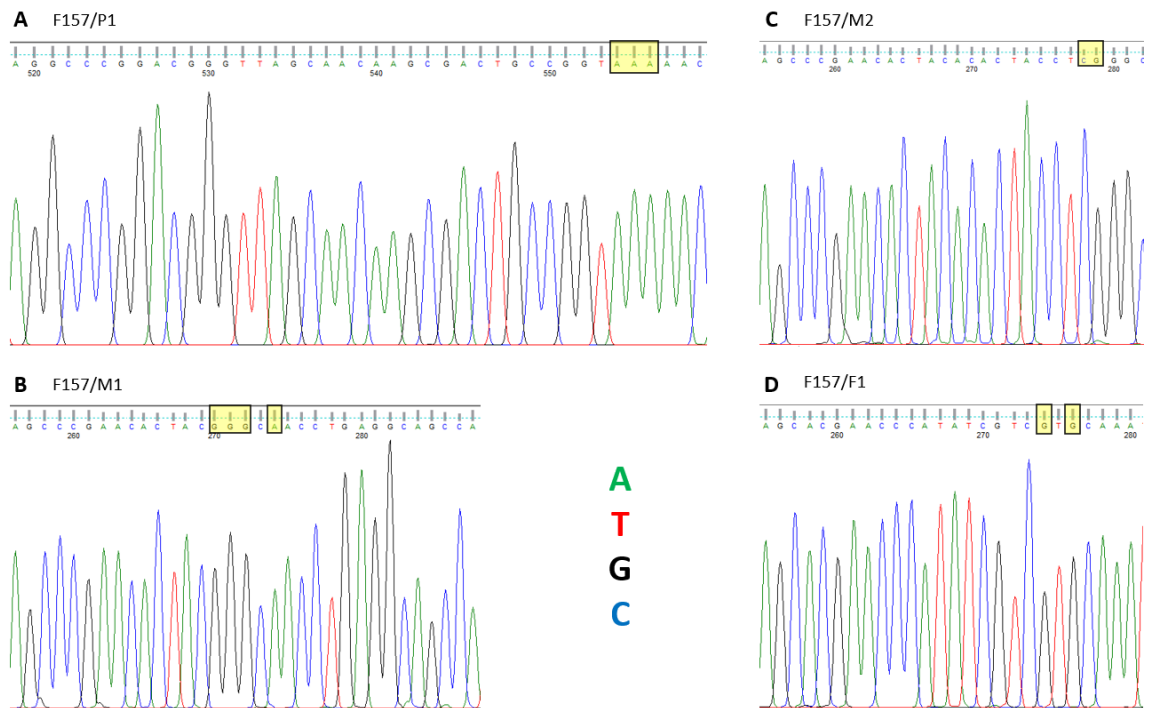


Figure 4.19 - Sequence traces of rescued viruses. RNA was isolated from Vero cells infected with F157/P1 (A), F157/M1 (B), F157/M2 (C), and F157/F1 (D) and, following RT-PCR, sequenced at the genome location that has been changed. The mutated bases are highlighted by yellow boxes above the sequencing trace. Also shown is the colour code for bases.

4.4.2 The levels of NP, P, V, and M proteins following IFN pre-treatment

Pre-treatment of cells with IFN reduces levels of PIV5 protein production in an IFIT1-dependent manner, with M being affected more than NP and P^{149,150,290}. To test whether the introduced changes in the P and M 5'UTRs affect their sensitivity to IFIT1, A549 and A549 IFIT1^{-/-} cells were infected with F157, F157/P1, F157/M1, and F157/M2 at an moi of 5 either with or without IFN treatment at 8h pre-infection, and at 18hpi the cell lysates were prepared for analysis by western blot. The F157/F1 virus was not investigated due to the lack of a suitable F antibody.

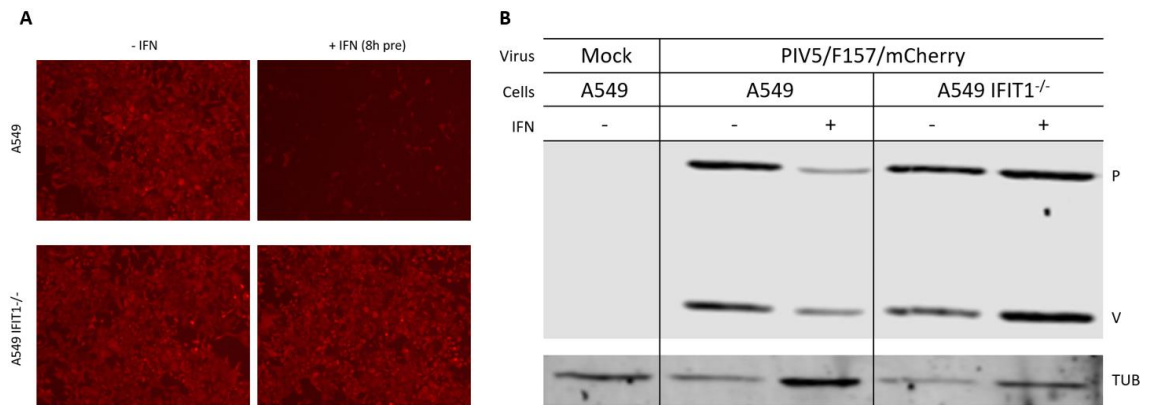


Figure 4.20 - IFIT1 reduces PIV5 protein synthesis. A) A549 and A549 IFIT1^{-/-} cells were or were not treated with IFN at 8h pre-infection, and then infected with PIV5/F157/mCherry at an moi of 5. Cells were imaged for mCherry at 18hpi with a constant exposure time. **B)** At 18hpi, the cells from panel A were harvested for SDS-PAGE and western blot. An equal amount of protein was loaded in each lane and proteins were detected with mouse anti-V5 and rabbit anti-tubulin. The V5 epitope is present in both the P and V proteins.

Firstly, the sensitivity of PIV5 protein synthesis to IFIT1 in A549 cells was confirmed by infection of either unedited or IFIT1^{-/-} cells with or without pre-treatment with IFN. Pre-treatment of cells with IFN greatly decreased the level of PIV5 protein synthesis, as detected by both mCherry intensity (figure 4.20A) and western blot for the P and V proteins (figure 4.20B). The mCherry 5'UTR consists of the first 26 bases of the P/V 5'UTR followed by 9 inserted bases upstream of the mCherry CDS. This truncated P/V UTR is predicted to form only a weak 5' structure with a 2-base overhang (see figure 4.18) and therefore mCherry translation is likely to be much more affected by IFIT1 than P/V. Both mCherry intensity and P/V protein levels were similar in IFN-treated and untreated knockout A549 cells, showing that the inhibitory effect of IFN on viral translation is solely due to IFIT1. IFN treatment had a noticeable effect on tubulin with an apparent induction in infected cells. This effect has been previously reported³²⁰ and means that tubulin is not an appropriate loading control for these experiments. To test the UTR mutant viruses, it was therefore decided to blot for NP in addition to P/V or M and use the ratio between the viral proteins to determine the relative effect of IFIT1.

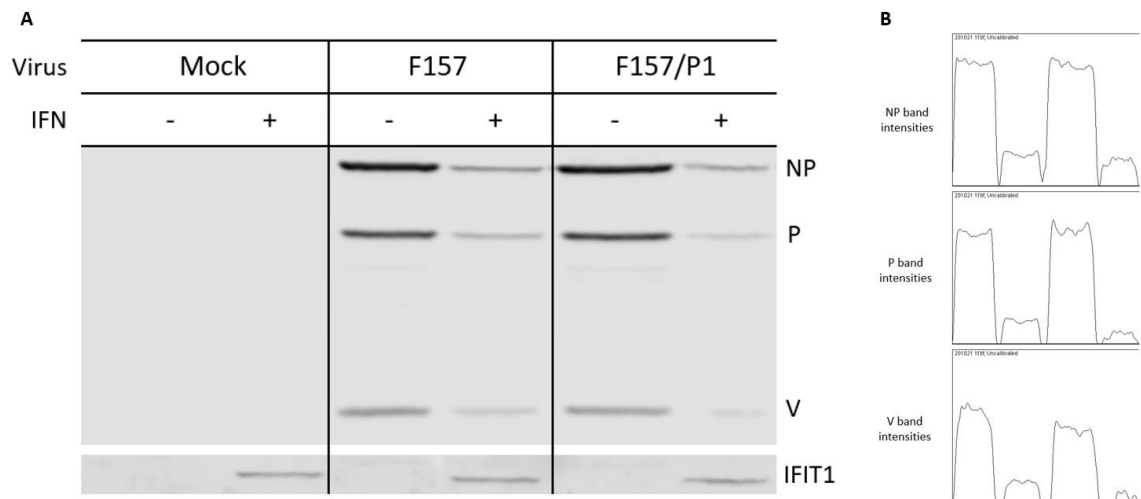


Figure 4.21 - The P1 UTR mutation increases sensitivity to IFIT1. A) A549 were or were not treated with IFN at 8h pre-infection, and then infected with F157 or F157/P1 at an moi of 5. At 18hpi, cells were harvested for SDS-PAGE and western blot. An equal amount of protein was loaded in each lane and proteins were detected with mouse anti-V5, mouse anti-NP, and rabbit anti-IFIT1. The V5 epitope is present in both the P and V proteins. **B)** The NP, P, and V band intensities for the last four lanes of panel A are shown, as generated using ImageJ software.

The F157/P1 virus was found to have a slightly increased sensitivity to IFN/IFIT1, as demonstrated by a small reduction in the level of P and V relative to the unchanged F157 virus following IFN pre-treatment (figure 4.21). The negative effect of the UTR mutation on P and V levels was quite small, suggesting that the 4nt overhang introduced by disrupting the RNA secondary structure is not sufficient to completely sensitise this UTR to IFIT1. A reduced level of P/V may also reduce the level of NP because P is required for viral transcription and V is required to dismantle the IFN-induced antiviral state by targeting STAT1 for degradation; this makes normalising the level of P/V to that of NP difficult.

Pre-treatment of A549 cells with IFN prior to infection with PIV5/F157/mCherry resulted in a complete loss of detectable M (figure 4.22). Any decrease in the sensitivity of M to IFIT1 will therefore be clearly seen and although no such result was seen with F157/M2, F157/M1 was found to produce a significant amount of M despite the presence of IFIT1. The F157/M1 virus differs from F157 by only four bases in the 5'UTR of M (figure 4.18) and does not differ in protein levels in the absence of IFN, so the difference in M protein levels can only be due to the altered structure of the 5'UTR which decreases sensitivity to IFIT1.

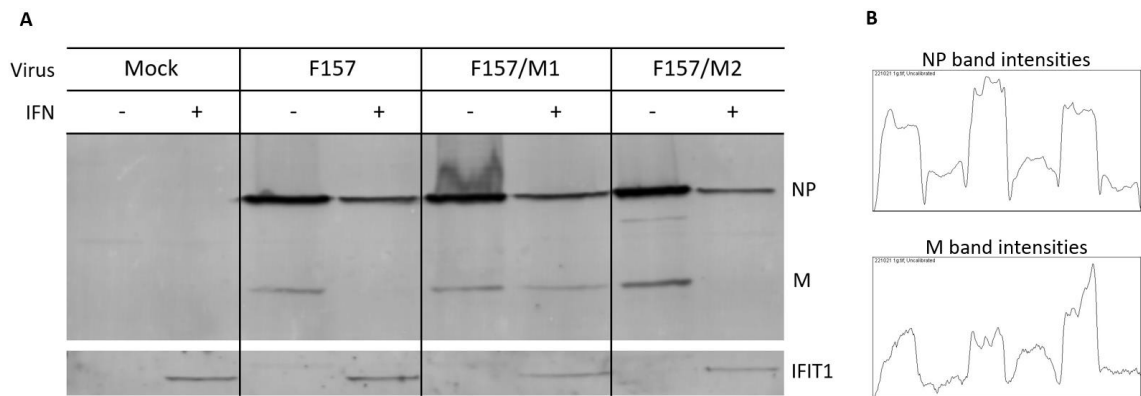


Figure 4.22 - The M1 UTR mutation decreases sensitivity to IFIT1. A) A549 were or were not treated with IFN at 8h pre-infection, and then infected with F157, F157/M1 or F157/M2 at an moi of 5. At 18hpi, cells were harvested for SDS-PAGE and western blot. An equal amount of protein was loaded in each lane and proteins were detected with mouse anti-M, mouse anti-NP, and rabbit anti-IFIT1. **B)** The NP and M band intensities for the last six lanes of panel A are shown, as generated using ImageJ software.

4.4.3 Growth curves of viruses with mutated 5'UTRs

To determine the impact of the altered levels of M on viral growth, growth curves were performed in A549 and A549 IFIT1^{-/-} cells with or without IFN pre-treatment, and viral titre determined at 12, 24, 48, and 72 hpi. F157/M1 was selected over F157/M2 because of the increased level of M protein in response to IFN shown in figure 4.22. It would also be interesting to investigate the effect of reduced P/V levels by performing a growth curve with F157/P1, but this was not undertaken due limited time and resources. The same applies to the untested F157/F1 virus.

The result of these growth curves with F157/M1 are shown both as a comparison between A549 and A549 IFIT1^{-/-} cells (figure 4.23 A-B) and as a comparison between the F157 and F157/M1 viruses (figure 4.23 C-D). The data in panel A showing that F157 is highly sensitive to IFN and that this is mostly (but not totally) due to IFIT1 was previously presented and discussed in chapter 3 (figure 3.35). In the absence of IFN, the growth kinetics of F157 and F157/M1 were indistinguishable (see panel C), showing that there is no fitness cost of increased M 5'UTR structure. However, when A549 cells were pre-treated with IFN, F157/M1 had a significant growth advantage over F157, with titres at both 48 and 72 hpi being approximately 10-fold greater (panel C). Importantly, no growth advantage was seen for F157/M1 in IFN-treated A549 IFIT1^{-/-} cells, as it grew to the same titre as F157 (panel D). This not only shows that the reduced IFN sensitivity of F157/M1 is due to reduced inhibition of translation by IFIT1, but also excludes the possibility that the results are affected by any unintended difference in moi between the viruses when infecting the cells.

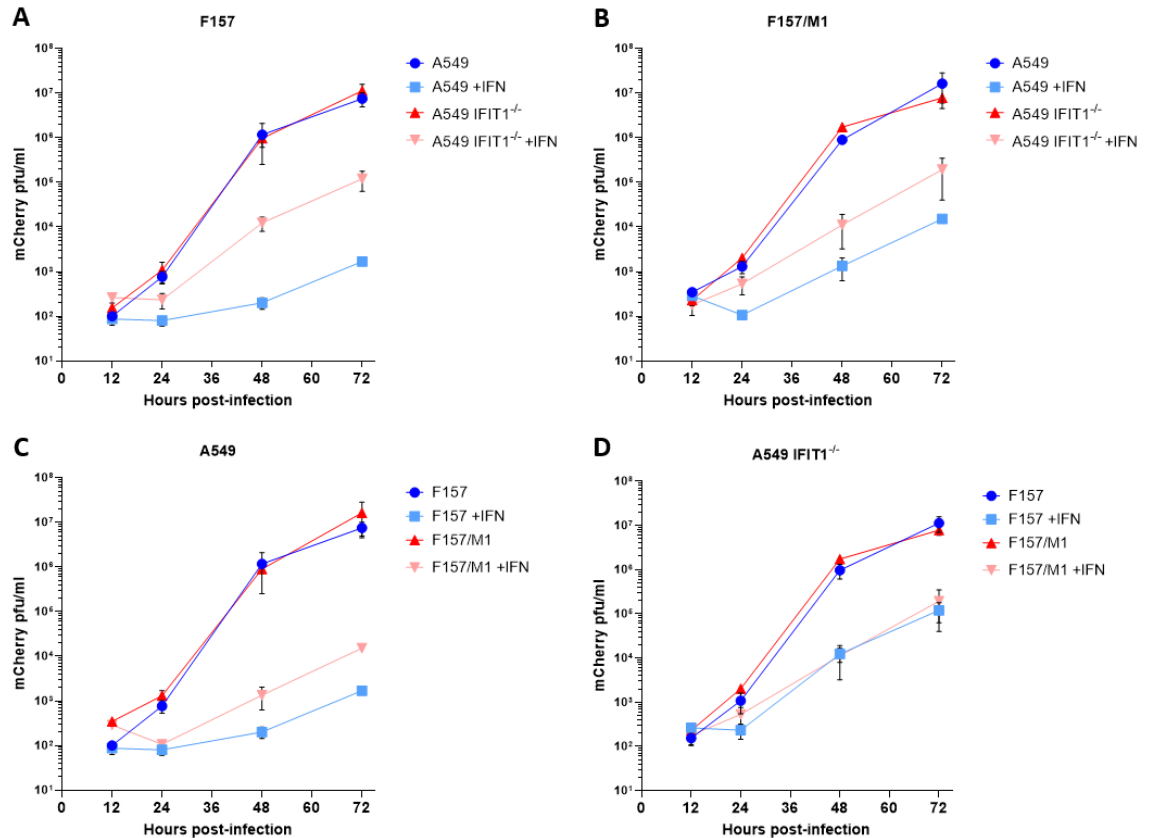


Figure 4.23 - Growth curves of PIV5/F157 (A) and PIV5/F157/M1 (B) in A549 and A549 IFIT1^{-/-} cells. Confluent monolayers of cells were or were not pre-treated with IFN at 8h pre-infection, and then infected with PIV5/F157/mCherry at an moi of 0.01. Cell supernatants were harvested at 12, 24, 48, and 72 hpi and flash-frozen. Samples were titrated by plaque assay in Vero cells and results presented as pfu/ml. C-D) The same data from panels A-B is presented by separation of A549 and A549 IFIT1^{-/-} rather than by virus. y-axis scale is logarithmic. Error bars are SEM and data are an average of three independent replicates. No error bars are shown when the bars are smaller than the symbol of the data point. The F157 data here were previously presented in chapter 3.

The altered 5'UTR of F157/M1 does not fully desensitise this virus to IFIT1, as shown in panel B. In fact, the approximately 10-fold increase in viral titre relative to F157 is surprising because although the level of M is higher, the F and HN 5'UTRs are unaltered and the level of these proteins would be expected to still be severely reduced by IFIT1. This demonstrates the importance of M and suggests that the level of this protein is the main limiting factor in viral assembly and particle production. The ability to introduce RNA secondary structure to limit the IFN-sensitivity of viruses which generate cap0 mRNA also has practical implications in the expression of transgenes from viral vectors. Both PIV5^{321,322} and alphaviruses^{323,324} are commonly used in candidate vaccines as vectors for the transgenic expression of antigens.

Despite the clear importance of M for viral growth, it would be interesting to test a virus with strong secondary structure introduced into the 5'UTRs of all genes in an attempt to further reduce its sensitivity to IFIT1. Assuming that this would indeed have the intended effect, it raises

the question of why the virus has not simply evolved to have strong structures similar to that of the P/V 5'UTR in all its genes so that its sensitivity to IFN and IFIT1 is minimised. It is notable that the genes most sensitive to IFIT1 are primarily involved in viral particle production (M, F, and HN), while the relatively insensitive NP and P/V genes are involved in genome maintenance, RNA synthesis, and immune evasion. It is possible that the virus has evolved to use sensitivity to IFIT1 as a mechanism of post-transcriptional gene regulation so that the relative levels of viral proteins are altered during the IFN-induced antiviral state in a way which favours genome maintenance over particle production. This idea is expanded upon in the discussion section.

4.5 Chapter Summary

The results in this chapter have shown that the different sequences of the 5'UTRs of PIV5 genes are responsible for the differential effect of IFIT1 on PIV5 protein levels during infection. The formation of RNA secondary structure at the 5' end of the NP and P/V mRNAs provides some protection from IFIT1, while the unstructured 5' end of the M mRNA leaves it completely sensitive. Changing downstream sequences of the 5'UTR to introduce secondary structure can decrease the sensitivity of M mRNA to IFIT1, and when incorporated into the virus these changes increase viral particle production in IFN-treated cells. The virus has possibly evolved these differing structures in the 5'UTRs of its genes as a form of post-transcriptional gene regulation dependent upon IFIT1, which may provide a counter-intuitive fitness advantage by, for example, promoting persistent infection.

Chapter 5 – A yeast growth assay to investigate mRNA cap-binding proteins

The work presented in the previous two chapters, in particular chapter 3, has highlighted some properties of IFIT proteins that warrant further investigation. hIFIT1 and mIFIT1 are both able to bind cap0 and inhibit the translation of mRNAs bearing this structure, but they differ in their affinity for capped RNA, their requirement for co-factors, and in the degree to which they inhibit the MGs of PIV5 and NDV. Most notable was the unexpected finding that chIFIT5 is able to inhibit the MGs of paramyxoviruses even when the ppp-binding protein hIFIT5 does not. As briefly discussed in chapter 3, these results imply that chIFIT5 is possibly acting upon capped mRNA, a property not previously reported for this avian IFIT.

This chapter uses a modified version of a published yeast growth assay¹⁶¹ to investigate the cap-binding properties of IFIT proteins. Two main questions are addressed:

- Do cap-proximal methylations (cap1 2'-O-methylation, cap2 2'-O-methylation, and N6-adenosine methylation) have differing effects on the affinity of hIFIT1 and mIFIT1 for mRNA cap structure?
- Is chicken IFIT5 able to bind RNA structures other than ppp-RNA, and could it fill the role of a cap-binding protein apparently missing in birds due to a lack of IFIT1¹⁶⁰?

The yeast growth assay is first validated and then used to test the above hypotheses by co-expression of IFITs with human mRNA cap MTases in yeast cells.

5.1 Validation of inhibition of yeast growth as an assay for mRNA cap-binding proteins

While higher eukaryotes generate cap1 and cap2 mRNA structures using the cap-specific 2'-O-methyltransferases CMTR1¹⁸⁶ and CMTR2¹⁸⁷, yeast lack these enzymes and therefore produce cap0 mRNA²⁵⁸. A recent study has taken advantage of this to investigate hIFIT1 and mIFIT1 by expressing them in *Saccharomyces cerevisiae* and using inhibition of yeast growth as a readout of cap0-binding¹⁶¹. Daugherty *et al.* were able to show that both hIFIT1 and mIFIT1 are toxic to yeast because they bind to cap0 structures and inhibit yeast mRNA translation. CMTR1 co-expression was able to completely rescue inhibition by mIFIT1. However, CMTR1 expression was insufficient to rescue yeast growth in the presence of hIFIT1, suggesting that hIFIT1 may also be

able to bind to cap1 structures to some extent. The authors conclude that hIFIT1 and mIFIT1 are paralogs which diverged over 100 million years ago and likely have different, though similar, functions.

The simplicity of transforming yeast to assess the cap0-binding ability of mammalian proteins makes it an attractive alternative to EMSAs and other more labour-intensive methods, at least as a preliminary investigation. Using *S. cerevisiae* strain PJ69-4 α , an attempt was made to establish such an assay and to replicate the results of Daugherty *et al.*¹⁶¹ While the previous study developed yeast strains with inducible expression of IFITs, here yeast have simply been transformed with IFITs expressed from a vector which also expresses a selectable marker; any negative effect on growth can be assessed by growing the transformants on selective agar plates.

Firstly, hIFIT1 and mIFIT1 were transformed into yeast to confirm that expression of these proteins does indeed affect growth of PJ69-4 α (figure 5.1). Compared to the control plate, expression of both hIFIT1 and mIFIT1 resulted in a dramatic reduction in the number of colonies at day 3 post-transformation. By days 5 and 7, the few colonies on these plates developed an unusual morphology with an apparent increased growth rate relative to the control plate colonies. This is possibly due to chance silencing of hIFIT1/mIFIT1 in these cells combined with either a reduced competition for nutrients encouraging an increased growth rate or reduced quorum sensing, a process which can regulate growth in response to changes in cell density³²⁵; this was not investigated further. What is clear is that expression of IFIT1 produces a distinct phenotype of dramatically reduced numbers of 'normal' colonies and that yeast transformation can potentially serve as an assay of cap0-binding proteins. To confirm this, the cap-binding mutations used in the previous chapters – hIFIT1(R187H) and mIFIT1(R184H) – were also tested. These changes in IFIT1 completely restored normal colony appearance and therefore the toxicity seen with hIFIT1 and mIFIT1 is most probably dependent on their cap-binding ability.

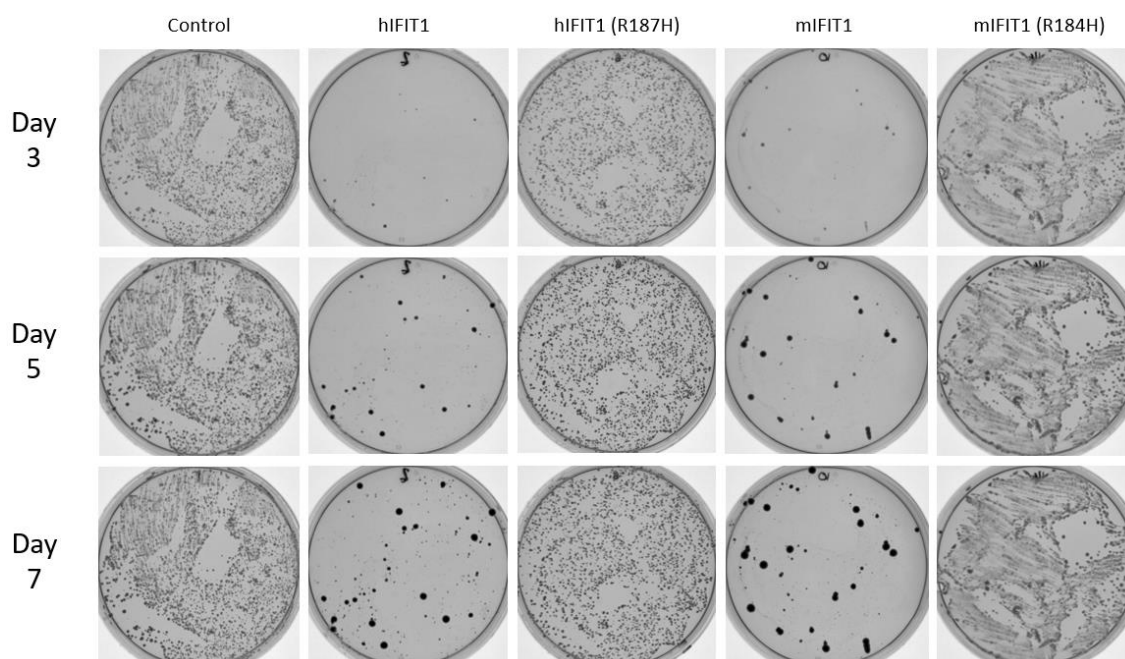


Figure 5.1 - Toxicity of hIFIT1 and mIFIT1 in yeast. Yeast strain PJ69-4a was transformed with pGADT7delGAL (Leu selection), pHON1delGAL (Trp selection), and pHON7 (Ura selection) and plated on SD –Leu –Trp –Ura agar. hIFIT1 and mIFIT1 were expressed from pHON7 in addition to mutants with characterised defects in cap and 5'-ppp binding (hIFIT1 R187H and mIFIT1 R184H). Plates were incubated at 30°C and imaged at 3, 5, and 7 days post-transformation.

In the published system in which IFITs are expressed as galactose-inducible transgenes, complete loss of yeast growth was seen with both mIFIT1 and hIFIT1. Multiple factors could explain the discrepancy between figure 5.1 and the previously published results, including differences between yeast strains, the amount of IFIT1 expressed, or that the expression vector for IFIT1 used here (pHON7) includes an N-terminal NLS (intended for use in yeast-3-hybrid assays) which may reduce the impact of IFIT1 on cytoplasmic translation. Despite this, transformation of yeast with IFITs, as shown above, produces a clear and useful phenotypic effect on colony count and morphology.

5.2 The potential role of cap-proximal methylations in protection from IFITs

Daugherty *et al.* found that co-expressing CMTR1 rescued yeast growth from mIFIT1 but not hIFIT1. The authors speculated that co-expressing both CMTR1 and CMTR2, to generate cap2, may offer protection from hIFIT1, but this was not tested. The potential for additional cap methylations to reduce IFIT1 binding is demonstrated by the reduced affinity of IFIT1 for the structure of the artificial anti-reverse cap analog (ARCA)³²⁶, in which the cap guanosine is 3'-O-methylated. Subsequent to the study of Daugherty *et al.*, cap-specific m6A methyltransferase

(CAPAM, previously called PCIF1) was identified as the human enzyme responsible for generating N6,2'-O-dimethyladenosine (m6Am) at the first nucleotide of A-initiating transcripts³²⁷. A significant portion of human transcripts are modified in this way²⁵⁸ and this methylation could affect the affinity of IFIT1 for mRNA cap structure by serving as an additional mRNA 'self' signal.

Given the discrepancy seen between hIFIT1 and mIFIT1 by Daugherty *et al.*, and the known impact of cap-proximal methylations on IFIT binding affinity, the ability of CMTR2 and CAPAM to rescue yeast growth from hIFIT1 and mIFIT1 was tested in combination with CMTR1. Firstly, CMTR1 alone was co-expressed in an attempt to replicate the results of Daugherty *et al.* (figure 5.2). CMTR1 co-expression resulted in restoration of normal colony morphology for mIFIT1 with increased numbers, loss of the unusual fast-growing colonies, and only a small delay in growth which was most apparent at day 3. This agrees with the previous report and shows that mIFIT1 is a cap0-binding protein with minimal affinity for cap1. The incomplete rescue of growth at day 3 is likely due to non-total cap methylation by CMTR1 which is to be expected given that this enzyme usually operates co-transcriptionally¹⁸⁶ and likely with additional factors in human cells, and will therefore be unlikely to achieve complete methylation of yeast mRNAs.

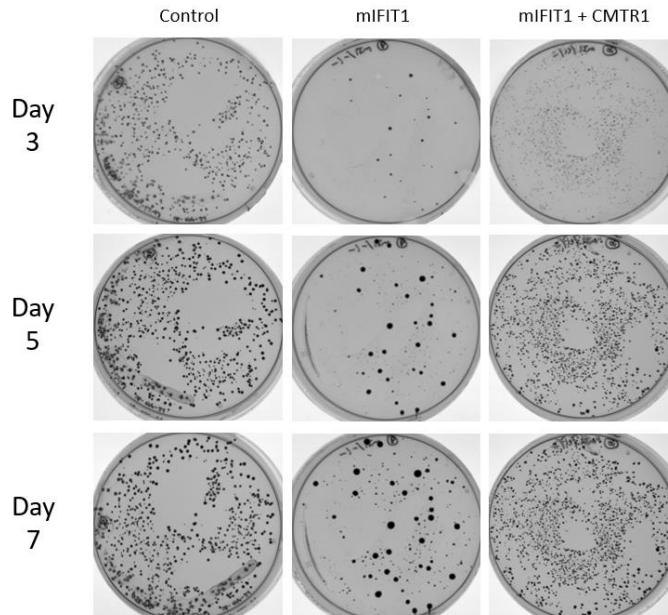


Figure 5.2 - Cap methylation reduces toxicity of mIFIT1 in yeast. Yeast strain PJ69-4 α was transformed with pGADT7delGAL (Leu selection), pHON1delGAL (Trp selection), and pHON7 (Ura selection) and plated on SD –Leu –Trp –Ura agar. mIFIT1 was expressed from pHON7 and CMTR1 co-expressed from pGADT7delGAL. Plates were incubated at 30°C and imaged at 3, 5, and 7 days post-transformation.

The experiment was then repeated with hIFIT1 and the additional MTases CAPAM and CMTR2 to investigate whether further cap-proximal methylations are required for protection from

hIFIT1. However, co-expression of CMTR1 alone was sufficient to significantly rescue yeast growth from hIFIT1 and to restore a near-normal colony morphology (figure 5.3). Although the rescue by CMTR1 for hIFIT1 is perhaps less than that for mIFIT1 (compare the day 3 plates for hIFIT1 and mIFIT1 in figures 5.2 and 5.3), this result differs from that seen by Daugherty *et al.* and makes it difficult to assess the impact of further cap methylations. Reasons for this difference could be due to the mode of expression, localisation, timepoint, or yeast strain, as previously discussed. Nevertheless, co-expression of CMTR1 and CMTR2 did appear to provide a small additional rescue of yeast growth from hIFIT1 compared to expression of CMTR1 alone, most notably on the day 3 plate. This suggests that cap2 mRNA may be less sensitive to hIFIT1 than cap1, as has been previously suggested¹⁸⁰, and that further modifications of mammalian cellular mRNA may provide an additional 'self' signal not present on RNA virus mRNAs since only large dsDNA viruses, such as African swine fever virus and Mimivirus, are known to encode orthologs of CMTR2¹⁸⁷.

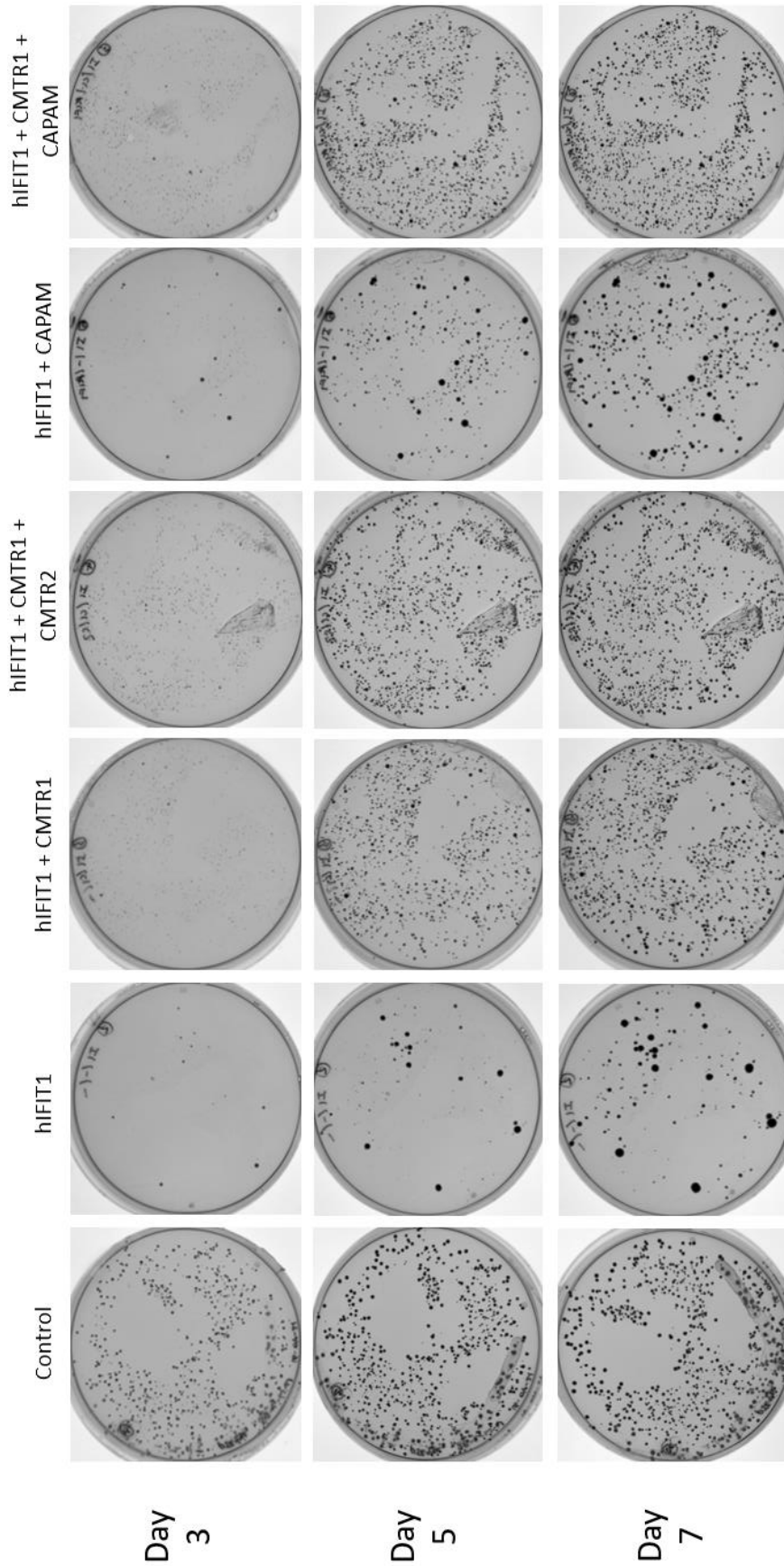


Figure 5.3 - Cap methylation reduces toxicity of hIFIT1 in yeast. Yeast strain PJ69-4 α was transformed with pGADT7delGAL (Leu selection), pHON1delGAL (Trp selection), and pHON7 (Ura selection) and plated on SD –Leu –Trp –Ura agar. hIFIT1 was expressed from pHON7 and was co-expressed with the indicated human methyltransferases (CMTR1 in pGADT7delGAL, CMTR2 and CAPAM in pHON1delGAL). Plates were incubated at 30°C and imaged at 3, 5, and 7 days post-transformation.

Interestingly, the small additional protection from hIFIT1 offered by CMTR2 was also conferred by co-expression of CAPAM with CMTR1. Co-expression of CAPAM alone also resulted in a small increase in the number of colonies at days 5 and 7 compared to hIFIT1 without any MTase (figure 5.3). Only 80% of *S. cerevisiae* transcripts start with an adenine²⁵⁸ and CAPAM has a strong preference (7.6-fold) for m7GpppAm as a substrate over the m7GpppA present in yeast cells³²⁸. Therefore, the modest rescue of growth achieved by CAPAM perhaps represents a significant effect of adenine N6-methylation on hIFIT1 binding. m7Gpppm6A cap structure is not present in mammalian cells, but m7Gpppm6Am may provide a 'self' signal that protects from hIFIT1. As with CMTR2, RNA viruses lack an enzyme analogous to CAPAM, so this modification would have to be performed by recruiting cellular MTases.

To confirm that the rescue of growth seen with CAPAM is dependent upon its cap-specific MTase activity, a catalytic mutant was tested (CAPAM_{SPPG}). CAPAM_{SPPG} contains the mutations N553S and F556G which have been shown to abolish CAPAM's catalytic activity³²⁸. Catalytic inactivation abolished the modest rescue of growth seen for both hIFIT1 (figure 5.4) and mIFIT1 (figure 5.5). The negative effect of the mutations on CAPAM activity was most obvious for mIFIT1 because CAPAM appears to provide greater rescue from mIFIT1 than hIFIT1, suggesting that mIFIT1 is less able to tolerate cap-proximal methylations than hIFIT1. This supports the conclusion of Daugherty *et al.* that hIFIT1 and mIFIT1 are paralogues with differing functions¹⁶¹ and also agrees with suggestions that hIFIT1 has a more relaxed specificity than does mIFIT1¹⁸⁰.

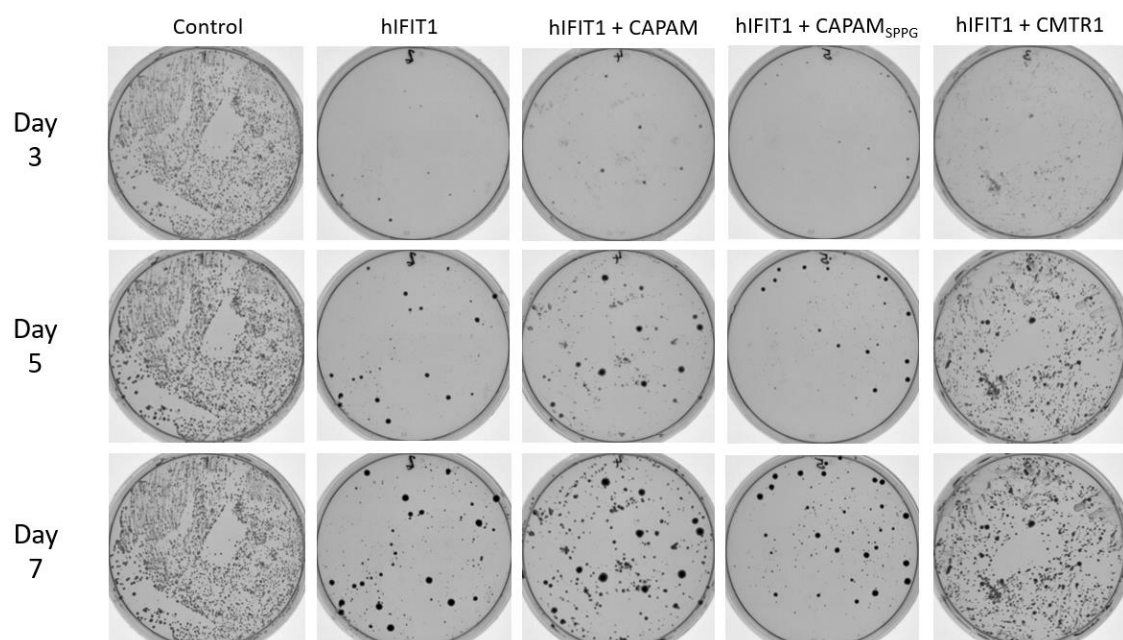


Figure 5.4 - Reduction of hIFIT1 toxicity in yeast by CAPAM is dependent upon its cap-specific m6A methyltransferase activity. Yeast strain PJ69-4α was transformed with pGADT7delGAL (Leu selection), pHON1delGAL (Trp selection), and pHON7 (Ura selection) and plated on SD –Leu –Trp –Ura agar. hIFIT1 was expressed from pHON7 and was co-expressed with the indicated human methyltransferases (CMTR1 in pGADT7delGAL, CAPAM in pHON1delGAL). 'SPPG' is a catalytically inactive mutant of CAPAM. Plates were incubated at 30°C and imaged at 3, 5, and 7 days post-transformation.

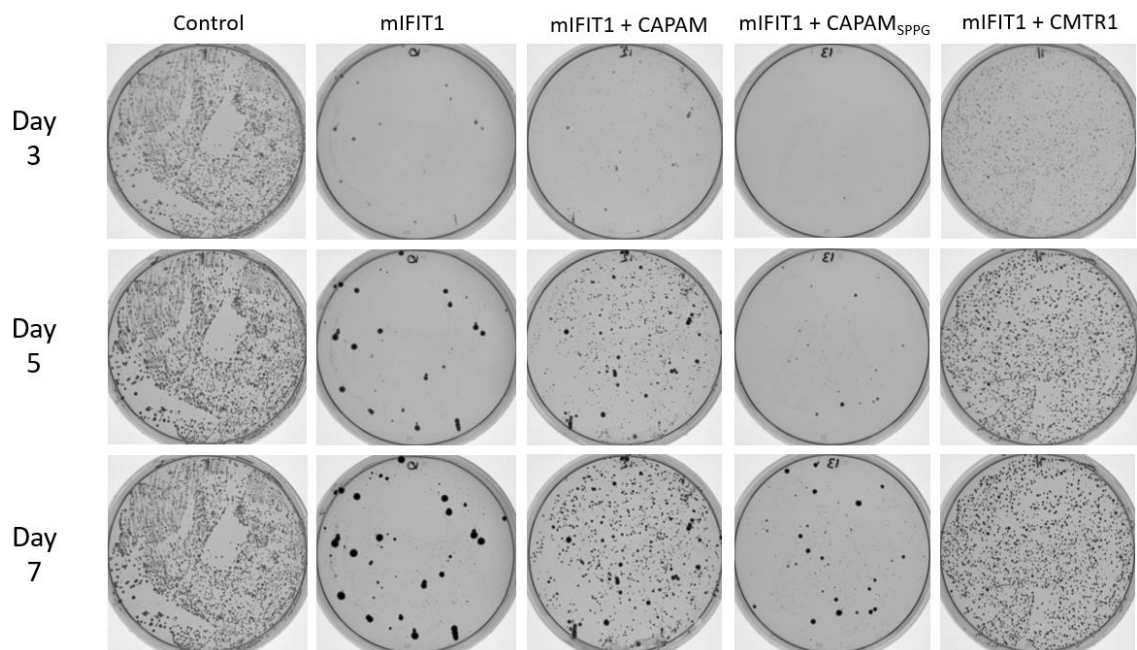


Figure 5.5 - Reduction of mIFIT1 toxicity in yeast is by CAPAM is dependent upon its cap-specific m6A methyltransferase activity. Yeast strain PJ69-4 α was transformed with pGADT7delGAL (Leu selection), pHON1delGAL (Trp selection), and pHON7 (Ura selection) and plated on SD –Leu –Trp –Ura agar. mIFIT1 was expressed from pHON7 and was co-expressed with the indicated human methyltransferases (CMTR1 in pGADT7delGAL, CAPAM in pHON1delGAL). ‘SPPG’ is a catalytically inactive mutant of CAPAM. Plates were incubated at 30°C and imaged at 3, 5, and 7 days post-transformation.

While many viruses encode enzymes capable of generating cap1 mRNA structure (e.g. *Paramyxoviridae* (excluding *Rubulavirus*), *Filoviridae*, *Rhabdoviridae*, *Poxviridae*), no known equivalents of CMTR2 or CAPAM exist in RNA viruses. Despite this, many cytoplasmic RNA viruses produce mRNA with methylations beyond cap1, such as DENV (m7Gpppm6Am)³²⁹ and VSV (m7Gpppm6AmAm)^{318,330}. These structures can only be generated by recruitment of host enzymes, as demonstrated by VSV mRNA transcribed by the viral polymerase from in vitro-purified virions generating m7GpppAm³³¹ without the additional m6Am and cap2 methylation seen on VSV mRNA in infected cells³³⁰. A recent report has provided an example of such recruitment by showing that CAPAM is required for m6Am modification of VSV mRNA and that this provides protection from the antiviral response induced by IFN³¹⁸. However, in a binding assay with recombinant hIFIT1 and hIFIT3, N6 methylation of the first transcribed base was found to only slightly reduce affinity. It would be interesting to repeat such an experiment with mIFIT1 given that CAPAM was better able to rescue yeast growth from mIFIT1 than hIFIT1 (figure 5.5). Tartell *et al.* conclude that there are likely ISGs other than IFITs that are responsible for the increased sensitivity of VSV to IFN in CAPAM knockout cells. Interestingly, the ratio of cap-proximal Am to m6Am varies widely between tissues, with the m6Am:Am ratio being particularly high in mouse brain tissue relative to liver²⁵⁹. In theory, immune privileged sites such as the brain could have high basal expression of an ISG which restricts translation of non-

adenine-N6-methylated transcripts. The evidence presented above which shows that CAPAM is able to methylate yeast transcripts potentially makes this a suitable model with which to screen potential cap-binding ISGs which detect non-CAPAM-modified transcripts.

5.3 Identification of potential chicken cap-binding proteins

Birds lack an IFIT1 or IFIT1B and many, for example chickens, encode a single IFIT5-like IFIT protein^{161,245}. Chickens also lack RIG-I³³², so the loss of a major innate immune gene would not be unprecedented, but the ubiquity of cap0-binding proteins in mammals and the important role they play in translational inhibition of non-self viral mRNA mean that it would be surprising if birds had not evolved a similar antiviral protein. In the absence of an ortholog of IFIT1, the most likely candidates were hypothesised to be chIFIT5 and chMDA5. The former has some affinity for ppp-RNA²⁴⁵ but has not been tested with capped RNA. The latter is a PRR which binds viral dsRNA but the human ortholog, hMDA5, has been suggested to additionally recognise cap0 mRNA³³³. The closely related hRIG-I has been more convincingly demonstrated to bind cap0, although this requires the 5'-terminal base to be base-paired^{276,277}.

chIFIT5 and chMDA5 were transformed into yeast along with hIFIT1 and mIFIT1 as cap0-binding controls and hIFIT5 as a ppp-RNA- (but not cap0-) binding control (figure 5.6). chIFIT5 produced a colony phenotype similar to that of hIFIT1 and mIFIT1 but with even fewer colonies at day 3 and lack of smaller colonies at day 7. Importantly, the ppp-binding protein hIFIT5 had no apparent toxic effect so the results seen with chIFIT5 are unlikely to be due simply to its ability to bind ppp-RNA. This result implicates chIFIT5 as a cap0-binding protein. In contrast, chMDA5 had no effect and is therefore unlikely to bind cap0 with high affinity. A negative result was also seen with hMDA5 which casts doubt on its proposed ability to sense cap0 mRNA during viral infection.

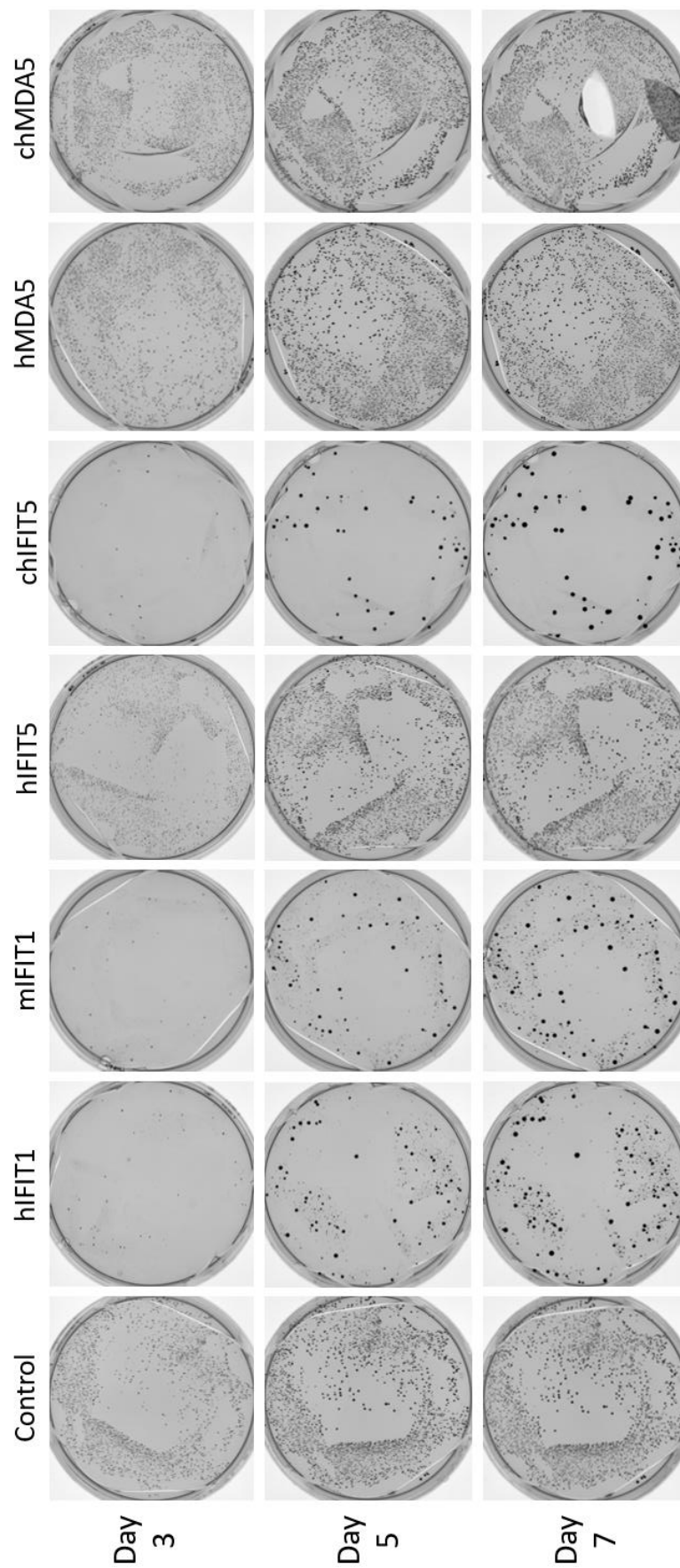


Figure 5.6 - *chIFIT5* is a potential *cap0*-binding protein. Yeast strain PJ69-4 α was transformed with pGADT7delGAL (Leu selection), pHON1delGAL (Trp selection), and pHON7 (Ura selection) and plated on SD –Leu –Trp –Ura agar. *hIFIT1*, *mIFIT1*, *hIFIT5*, *chIFIT5*, *hMDA5*, and *chMDA5* were expressed from pHON7. Plates were incubated at 30°C and imaged at 3, 5, and 7 days post-transformation.

Having identified chIFIT5 as a candidate cap0-binding protein, the effect of cap-proximal methylations on its affinity for RNA was tested. CMTR1 alone, or in combination with either CMTR2 or CAPAM, was co-expressed with chIFIT5, and colonies imaged as before (figure 5.7). Neither CMTR1 nor CAPAM alone were sufficient to restore yeast growth and even slower growth was seen with CMTR1 than without. Some level of normal growth was restored only when CMTR1 was co-expressed with either CAPAM or CMTR2, though this still took until days 5 and 7 to become apparent. These results suggest that chIFIT5 not only binds cap0, but can also bind cap1 with perhaps even greater affinity. This would not be without precedent because mIFIT1B also binds cap1 RNA²⁴⁷, but the potentially multifunctional nature of chIFIT5 (binding ppp, cap0, and cap1 RNA) would be unique and perhaps a result of the absence of other IFIT family members in birds¹⁶⁰.

The proposed binding properties of the IFIT proteins investigated in this chapter, as informed by results from the yeast growth assay and published analyses, are shown in figure 5.8.

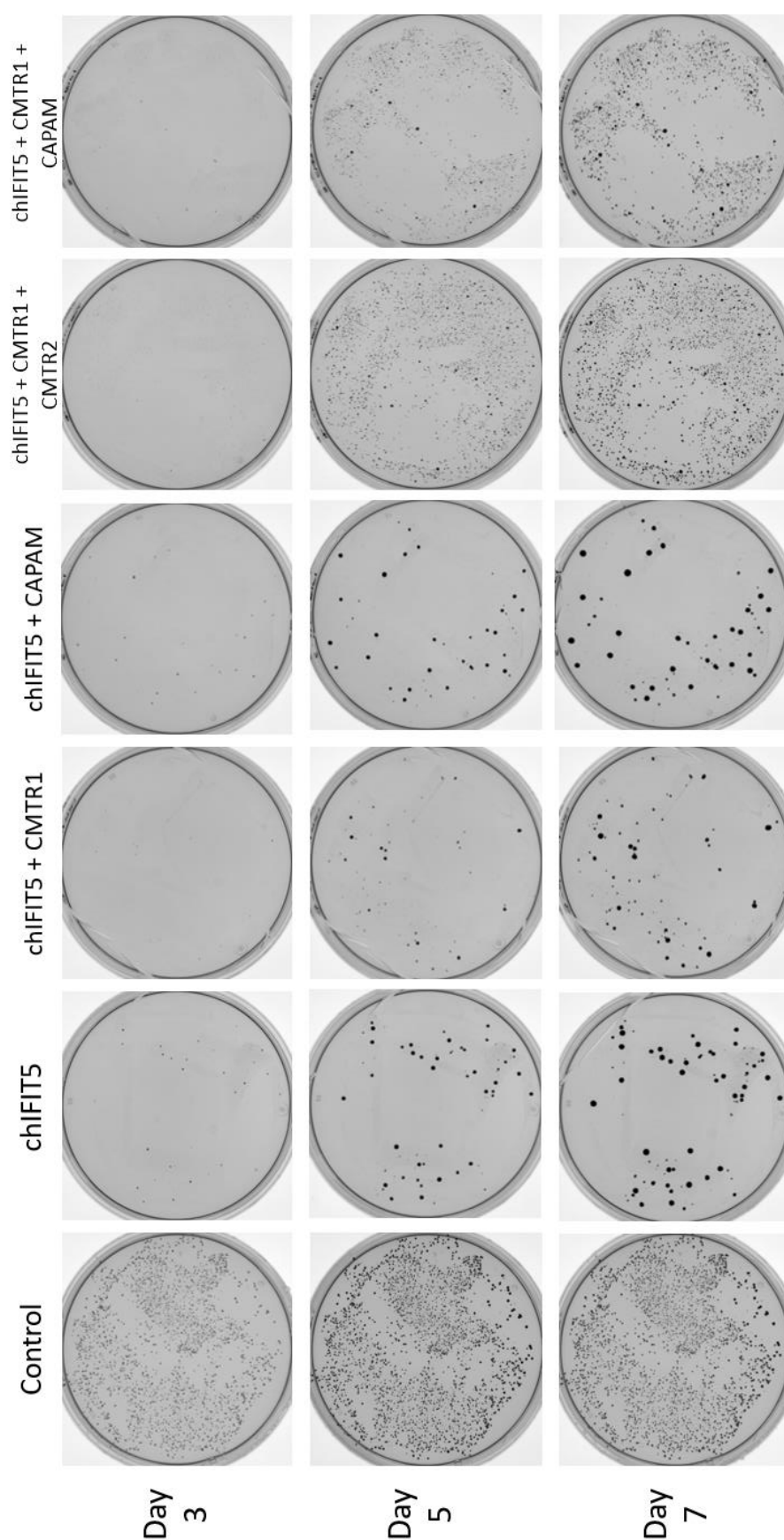


Figure 5.7 - chIFIT5 toxicity is only reduced by methylations beyond *cap1*. Yeast strain PJ69-4 α was transformed with *pGADT7delGAL* (Leu selection), *pHON1delGAL* (Trp selection), and *pHON7* (Ura selection) and plated on SD –Leu –Trp –Ura agar. chIFIT5 was expressed from *pHON7* and was co-expressed with the indicated human methyltransferases (CMTR1 in *pGADT7delGAL*, CMTR2 and CAPAM in *pHON1delGAL*). Plates were incubated at 30°C and imaged at 3, 5, and 7 days post-transformation.

	ppp	cap0	cap0/m6A	cap1	cap1/m6A	cap2
hIFIT1	+ ¹⁵²	+ ¹⁸⁰	-/+ ³¹⁸	-/+ ^{161,180}	-	- ¹⁸⁰
hIFIT1(R187H)	- ¹⁸⁰	- ¹⁸⁰	-	-	-	-
hIFIT5	+ ^{177,243}	- ¹⁷⁷	-	-	-	-
mIFIT1	-/+ ²⁴⁷	+ ^{130,247}	-/+	- ²⁴⁷	-	- ²⁴⁷
mIFIT1(R184H)	-	-	-	-	-	-
chIFIT5	+ ²⁴⁵	+	+	+	-	-

Figure 5.8 - The proposed RNA-binding properties of the IFIT proteins investigated in this chapter. These inferences have been made by combining the results seen in the yeast growth assay with published analyses of the proteins. + indicates high affinity, - indicates minimal affinity, and -/+ indicates a small or ambiguous affinity. Where a reference exists for the suggested binding property of each IFIT, a citation has been made.

5.4 Other avian IFIT proteins

chIFIT5 remains the only avian IFIT to have been investigated to any extent. Most birds have an IFIT5-like gene but some, like zebra finch, have an additional IFIT^{160,161}. This IFIT of unknown function (zfIFITx) lacks a conserved arginine residue (figure 5.9) which is conserved across all known cap0-binding IFIT proteins (compare to the alignment of IFIT proteins shown in chapter 3). The absence of this arginine suggests that either zfIFITx is unable to bind cap structures, or that it can bind cap1 but not cap0 (the cap1-binding protein mIFIT1B²⁴⁷ also lacks this arginine). One possibility is that zfIFITx regulates the activity of zfIFIT5 in mechanism analogous to that of human^{244,253} and murine²⁴⁷ IFIT proteins. However, the apparent loss in birds of the C-terminal YxxxL motif (figure 5.9, compare to the alignment in chapter 3), which mediates the interaction of hIFIT1 and hIFIT3^{162,244,253}, means that the details of this mechanism would likely differ from that described for mammalian IFITs.

An obvious extension to the work presented in this chapter would be to expand the analysis of avian IFITs to proteins other than chIFIT5. The yeast growth assay provides a simple means with which to determine whether these proteins can bind mRNA cap structures and whether the uncharacterised zebra finch IFITs work cooperatively. The investigation of avian IFIT proteins is an area of ongoing research.

chIFIT5	-----MSTISKNSLKNLLQLECYFTWTLK	26	
zfIFIT5	MRVGPINKMEGITAGSSTPLALECLKTSEQRRKTMSTISKDSLKISLLQLECHFTWALLK	60	
zfIFITx	-----MLPAAKLSKEQLKEKLDALQCHFTWMLGV	29	
	:.:**:.**.**:*** *		
chIFIT5	EDVDLSLEESIEDQIEFFI-KPNISNYNLLSYVYHLKLSDEEALEYLKAAEEIIRKYYP	85	
zfIFIT5	RHVSLEVEETILDHIKFLS-EYEIRDYNNLSYVNYLKNSEKALENLKAAEAIQKRHP	119	
zfIFITx	ESHSPQHLLQKLDVEIKHTAHQNQLALLGLQAYLHQLNNQSEALQSLRAAEHKEDE-Q	88	
	: . : * :. : . : : : . : : * : . : * : * : . :		
chIFIT5	GEIDRRSLVTWGNAYWIYYHMGRYEEAQQVYINKVENSCKKLSNTAHLKIQLPEIYAEQGF	145	
zfIFIT5	DEIDWRSVLTWGNAYWIYYHMQRYEEAQTYSKVENSCCKLSTAHGKIQLPEIYAEQGF	179	
zfIFITx	LASTAGSLIICGNAYWIHYLQGSYQEAETCLERVQQLCPT---PWDVRLIPHIAQKQG	144	
	** : ***** : * * : . : : * : . : * : * : *		
chIFIT5	ALLFGGKYNNRAKECFKNALREEPNNPEFNAGYAIAYVLEEFYSYRCEVDSSLEPLK	205	
zfIFIT5	ALLRFGFNYFERAKNCFEAMKSEPDNPDFNAGYATAMYLEVNQCWYRGETTPCLEALK	239	
zfIFITx	SLAIRARNGERARECFNMALMLPQNRSFRTGLAMAFYSWN-SSWQDTEREVRIQLE	203	
	: * : . : * : * : * : * : * : * : * : *		
chIFIT5	RALKLNPMDFYLLALLALKLQDSQVDEAEKCI--EGMKKTPYLPYFLRYAAKFYRRKK	263	
zfIFIT5	RAVELNPKDTSLAALLALVLQRLHRGHEGERYIQ--EGLQKTPDFVFLRHAANFYRRKG	297	
zfIFITx	RIVNEQPDNYKAKIYLARLLEQV---DGEKSIQGLAKECAEKSSDPEVLKLSALFWM-FR	258	
	* : : * : * : * : . : * : : . : . : * : : * : *		
chIFIT5	ELDKAQEVLERALEISPKSTFLHQLGLCYRAKLYELKNSTRYPQDQIEELIQICISHF	323	
zfIFIT5	EVDKAVEILKKALALTPKSVVLHHQLGLCYKFKALQLKK-TRHSPQEEAMNFMLAIFHF	356	
zfIFITx	STERALEIIQALQOEPGYHLLYQALANCYKQGWKAKEEKDKI-----RCKAIKDL	311	
	. : : * : * : * : * : * : * : * : * : * : *		
chIFIT5	KVVTQKPKFFSALI--DLARMYAEANMYRKAETFORALNVNITCSNKQEIYFYGNF	381	
zfIFIT5	KTVIDKKPVFFYAYS--DLAKAYAIKRYEEAEIYQKVLQKNDMAEEKQEIYLYNAYF	414	
zfIFITx	QKIVKTHPDLDTLAKLQLAEFIGAENPAKEKEIYMELERKINTLSLRQALSLSWGKY	371	
	: . : * : : : * : . : : * : : : * : : * : : . : :		
chIFIT5	LQYKKKSESEAIKYYKEGLKNGNYCFAE-KIRQYLKRLLEKRIQGLGGEEDFSTLGLIH	440	
zfIFIT5	QQFHMKSESAIKYYIECLKVEADSYARKKCEAVETLLKQKIESGLGDATDIGTLGLVH	474	
zfIFITx	FLYRDKSPEKAKAFMECYSIPLQTDHRQDCGRRLVKMAGIY--KSKGNTQ---AASAIH	426	
	: : * * . : * : * : . : * . : . : *		
chIFIT5	KLDGEKLEAIECYEKANEYNPDNEEYLSVLELRLSLSS	479	
zfIFIT5	NLNGKKQEAIECYEKALALDPNNEEYLNLAELQLSISS	513	
zfIFITx	RFF-----QEAHRHCPRNQL-HSV-----	444	
	. : : * * * : . :		
1: chIFIT5	100.00	58.37	25.52
2: zfIFIT5	58.37	100.00	25.80
3: zfIFITx	25.52	25.80	100.00

Figure 5.9 - Protein alignment of the chicken and zebra finch IFITs. Selected residues are highlighted in yellow, including the arginine at position 185 in chIFIT5 which is conserved across all known cap0-binding IFIT proteins (see alignment in chapter 3). Alignment was made with Clustal Omega. GenBank reference number for each IFIT protein can be found in the methods (section 2.5).

5.5 Chapter summary

The cap0 structure of yeast mRNA allows for the inhibition of yeast growth to serve as an assay for RNA-binding by IFIT proteins. Using this assay, cap-proximal methylations by CAPAM and CMTR1/2 are shown to reduce the affinity of mRNA cap structures for hIFIT1 and mIFIT1. chIFIT5 is able to bind multiple structures (ppp, cap0, and cap1), making it unique amongst all characterised IFIT proteins. The ability of chIFIT5 to bind cap1 mRNA likely serves as a strong host restriction factor for cytoplasmic RNA viruses, which do not encode cap2 MTases and would therefore have to recruit host MTases to evade the inhibitory activity of chIFIT5. The implications of this on both paramyxovirus evolution and the host range of avian RNA viruses are discussed in the next chapter.

Chapter 6 – Discussion

As mentioned in the introduction chapter, during the course of this project it became apparent that both themes (the mutations in the P protein of PIV5 and the sensitivity of PIV5 to IFIT1) are connected by their potential role in the ability of PIV5 to cause persistent infections. The short summaries at the end of each results chapter have given a brief overview of the main conclusions of the work, but how the findings may impact our understanding of how PIV5 replicates and interacts with the IFN system were not discussed in detail. Therefore, this chapter expands upon ideas mentioned in previous chapters and presents speculative theories on how reversible polymerase changes and selective sensitivity to IFIT1 may facilitate persistent infection, as well as the potential evolutionary origins and consequences of paramyxoviral sensitivity to IFITs.

6.1 Single amino acid changes in the PIV5 P protein as a potential mechanism of persistent infection

Single amino acid changes in the P protein of PIV5 affect the phenotype of the virus by increasing or decreasing the rate of viral transcription. Because these changes have been identified as naturally-occurring variants in different PIV5 strains, and are encoded by codons which vary by a single nucleotide, we have proposed that variation at these residues, particularly at amino acid 157, can serve as a ‘switch’ which shifts the virus from a ‘high-activity’, acute mode of infection to a ‘low-activity’, persistent one²²⁴. In this model, initial infection is likely to be with a ‘high activity’ variant, such as one which encodes F157 in P, because a higher rate of RNA synthesis causes increased cytopathic effect (CPE)^{224,334,335} and is more likely to induce symptoms (for example, ‘kennel cough’ in dogs caused the F157-encoding strain CPI+^{70,71}) conducive to spreading of the virus to new hosts. Following activation of the adaptive immune response, S157-encoding variants may be selected for because the reduced level of transcription caused by this change in P will limit the production of viral proteins and prevent clearance of infected cells by cytotoxic T cells. Having entered this low-activity state, the viral genome will be maintained by a low level of replication, but CPE and the production of viral particles will be limited, and the infection is likely to be asymptomatic. Periodically, a mutation will arise leading to an S157F change in the P protein. If the adaptive immune response has waned, this F157

variant will once again have a replicative advantage; viral particle production will increase, symptoms will return, and the infection can be passed onto a new host to repeat the cycle. Although such changes in P have been observed as minor variants during passage of the virus in cell culture⁶⁹, it would be interesting to obtain more sequencing data from in vivo infections of PIV5 to more conclusively demonstrate the existence of this mechanism of persistence.

The benefits to the virus of establishing a persistent infection are obvious. In fact, for RNA viruses that replicate quickly and have a high basic reproduction number (for example, MV has an R_0 of 12-18³³⁶), establishment of persistent infection in at least a minority of infected individuals may be a necessity to avoid the extinction of the virus due to a lack of new susceptible hosts. Persistent infection is most commonly associated with members of the dsDNA *Herpesviridae* family, with minimal focus on the potential for RNA virus persistence beyond the examples of HIV and HCV, which prolong infection by quasispecies-mediated evasion of the adaptive immune response⁷⁸⁻⁸⁰. In contrast to the high rate of HCV and HIV replication which generates a range of escape mutants to evade adaptive immunity, the proposed mechanism of persistence of PIV5 is more analogous to the latency of herpesviruses in that the virus fluctuates between a quiescent, latent state and an active, replicative one. Despite the instability of RNA relative to DNA, and the rapid turnover of RNA due to ubiquitous RNases, encapsidation of *Mononegavirales* genomes by the viral nucleoprotein (NP) renders them exceptionally resistant to degradation²³⁰. Nevertheless, in contrast to the episomal existence of herpesvirus genomes in the nucleus during latency³³⁷, RNA virus genomes are likely to require at least some level of ongoing replication, especially if selection of high-activity P variants is required for reactivation. Therefore, such infections are perhaps best termed 'pseudo-latent'. The mechanisms and consequences of persistent infection by RNA viruses have been recently reviewed^{77,338}.

The underappreciation of persistent infections by RNA viruses is perhaps exacerbated by the fact that most viral infections are not specifically diagnosed, and that a virus employing a pseudo-latent strategy is likely to cause only mild symptoms. It is therefore usually not possible to distinguish between a minor respiratory infection caused by a de novo infection and one caused by reactivation of a pseudo-latent virus. The extended periods of isolation endured by small groups of people at research stations in Antarctica provides a unique opportunity to observe the potential reactivation of RNA virus infections, and indeed 'outbreaks' of paramyxoviruses have been confirmed after multiple months of isolation³³⁹⁻³⁴². Despite imaginative claims of how such viruses may have been introduced to an isolated community³⁴³, the shedding of paramyxoviruses after such a long period of isolation instead provides strong evidence that these viruses are able to persist in humans in a pseudo-latent state. Reversible downregulation of RNA synthesis may be one mechanism by which pseudo-latency is achieved.

6.2 The loss of 2'-O-methyltransferase activity in the L proteins of rubulaviruses and avulaviruses, and the implications of chIFIT5 being a cap-binding protein

With the exception of alphaviruses of the *Togaviridae* family, and rubulaviruses and avulaviruses of the *Paramyxoviridae* family, all known human RNA viruses either generate cap1 structure on their mRNAs or, in the case of *Picornaviridae*, *Caliciviridae*, and *Astroviridae*, encode a genome-linked viral protein (VPg) which masks the 5' end of the genome. The failure of rubulaviruses and avulaviruses to generate cap1 mRNA structure is anomalous and requires explanation. One of the primary evolutionary functions of cap1/2 methylations in eukaryotes has been suggested to be the ability to discriminate between host, 'self', cap1 mRNA and viral, 'non-self', cap0 mRNA. As the innate immune proteins with the highest known affinity for cap0 mRNA, IFITs have likely provided the selective pressure for RNA viruses to evolve cap1 MTase activity (or to simply acquire cap1 structure by cap-snatching mechanisms, as for *Orthomyxoviridae* and *Bunyavirales*). Therefore, the loss of cap1 MTase activity in the L proteins of the closely related *Rubulavirus* and *Avulavirus* genera is perhaps indicative of the removal of this selective pressure. Due to the absence of IFIT1 in birds, one potential scenario is that a common ancestor of these genera became established in birds and lost cap1 MTase activity due to a lack of positive selection by IFIT1 restriction, or by negative selection of a redundant enzymatic activity which could slow replication kinetics. The reestablishment of these viruses in mammals (potentially forming what is now the *Rubulavirus* genus) would leave them without the ability to generate cap0 structure and thus sensitive to IFIT1.

The finding that chIFIT5 is not simply a ppp-binding protein but can also bind cap0 and cap1 structures is consistent with the proposal made above. There would be no selective pressure for a paramyxovirus to maintain its cap1 MTase activity in cells in which the translation of both cap0 and cap1 are inhibited to a similar extent. In fact, the effect of chIFIT5 on yeast growth (chapter 5) was marginally more severe when CMTR1 was co-expressed, suggesting that the affinity of chIFIT5 for cap1 may be even higher than for cap0; this would actually provide a strong selective pressure for viruses to lose cap1 MTase activity. Of note is that Infectious bronchitis virus (*Coronaviridae*), an RNA virus which infects chickens, may generate cap1 structures with a reduced efficiency compared to mammalian coronaviruses³⁴⁴.

For viruses infecting chicken cells, it would appear that the only viable strategies to evade restriction by chIFIT5 would be to either evolve 5'-proximal RNA structure (assuming that chIFIT5

is as affected by RNA structure as IFIT1 is) or to recruit host MTases to generate either cap2 or cap1/m6A mRNA structure. Regarding the first strategy, NDV 5'UTRs all contain a conserved sequence at the 5' end (8 bases, see figure 4.1) and are predicted to form extremely strong hairpin structures (with 1- or 2-base 5' overhangs) involving sequences as far downstream as position 77 (not shown), which have perhaps evolved to evade restriction by chIFIT5. Regarding the second strategy, cap1/m6A has been shown to be generated on VSV transcripts in human cells by recruitment of the host CAPAM enzyme³¹⁸ and it would be interesting to see if a similar recruitment of host MTases occurs during infection of chicken cells by NDV or other avian viruses. CAP-MAP analysis (as used for PIV5 in chapter 3) of NDV mRNA isolated from chicken cells would be able to show whether m6Am is generated at the first position, but the limitations of this method mean that cap2 methylation cannot be detected. Also of interest would be the cap structure of chicken cellular mRNA; the ratio of cap2 to cap1 (or of m6Am to Am) may prove to be much greater than for mammalian cells so that host translation is not affected by chIFIT5. Despici and Jaffrey have recently reported that mammalian CMTR2 generates cap2 with low efficiency so that only around 50% of cellular transcripts bear cap2 methylation¹⁹⁰; avian CMTR2 might be expected to be more efficient.

A more in-depth study of the MTase domain of rubulavirus L proteins is clearly needed to elucidate the mechanisms by which mRNA cap methylation is achieved and to explain the apparently defective 2'-O-MTase activity. A recent study has determined the structure of the PIV5 L-P complex and found an unusual conformation of the MTase and C-terminal domains of L³⁶, but more thorough structural and mutagenesis analyses, as have been performed for VSV and SeV, are required.

6.3 A possible role for the differential sensitivity of genes to IFIT1 in PIV5 persistent infection

With the previously mentioned exceptions, all known human viruses have evolved mechanisms to evade restriction of viral translation by IFIT1. Examples include the encoding of a cap1 MTase, the use of host enzymes for transcription and cap methylation, cap-snatching, and cap-independent translation from IRESs or VPg proteins. The apparent ease with which viruses evade inhibition by IFIT1 emphasises the uniqueness of rubulaviruses and avulaviruses, which are sensitive to IFIT1 for reasons speculated upon above.

Paradoxically, the absence of any of the evasion mechanisms mentioned in the previous paragraph could in theory provide an opportunity to utilise sensitivity to IFIT1 in some way to the benefit of this virus. Without the ability to generate cap1 mRNA structure, viruses can only reduce the affinity of IFIT1 for their transcripts by evolving to form cap-proximal RNA secondary structure. The effectiveness of this evasion strategy is demonstrated by the low affinity of the highly structured PIV5 P 5'UTR for IFIT1. Should there be a benefit to the virus of altering the relative levels of viral proteins during an IFN-induced antiviral state, then variation in 5'UTR structure between genes may evolve so that the level of some viral proteins are less affected by IFIT1 than others; in such a scenario, the virus would be utilising IFIT1 as a form of post-transcriptional gene regulation.

The use of IFIT1 as a form of post-transcriptional gene regulation during the IFN-induced antiviral state could explain why PIV5 has not evolved a structured M 5'UTR. At first, this seems counter-intuitive because the neighbouring P gene has evolved a strong hairpin which reduces IFIT1 binding and introduction of structure into the M 5'UTR reduces the inhibition of viral particle production by IFN (chapter 4). Why does the M 5'UTR not simply adopt a structure similar to that of the P 5'UTR? One explanation is that the reduced growth kinetics of the virus (dependent upon the induction of IFN) promotes prolonged, persistent infections in a similar manner to the proposed mechanism of low-activity variants of the P protein. However, the clear difference in sensitivity to IFIT1 between the PIV5 structural (M, F, and HN) and non-structural (NP and P/V) genes points to a more elegant mechanism.

Changes in the M proteins of *Mononegavirales* have been linked to alterations in viral phenotype from acute, lytic infections to persistent, non-lytic ones. The best example of this is subacute sclerosing panencephalitis (SSPE), a fatal condition caused by persistent Measles virus infection in which symptoms only present many years after initial infection. Despite the presence of MV RNA in the brain of patients who have died of SSPE, infectious virus particles are usually absent and the viral genome contains multiple mutations, particularly in the M gene^{345,346}. Many of these changes render the M protein non-functional due to a loss of NP-binding^{347,348} and the gene is often hyper-mutated to the extent that the protein is no longer produced^{349,350}. While the mechanisms enabling MV to persist and enter the CNS are not fully understood, the frequency with which M is hyper-mutated in cases of SSPE suggest that its loss may be a prerequisite for the persistence of MV in the CNS^{351,352}. The potential negative impact of M on viral persistence is supported by evidence from other *Mononegavirales* such as SeV (temperature sensitive mutations in M promote persistence^{353–355}) and VSV (changes in M promote persistence by reducing the induction of apoptosis³⁵⁶ and limiting CPE³⁵⁷).

The M protein of paramyxoviruses mediates the assembly and release of the virion by interacting with the cytoplasmic domains of the cell-surface viral glycoproteins, and relocating the encapsidated viral genome to the cell membrane via its interaction with NP^{56,358}. In the absence of M, replication and transcription of RNA can continue, but no viral particles will be formed, as demonstrated by the absolute requirement for M in the formation of virus-like particles. The IFIT1-dependent translational inhibition of PIV5 M during the IFN-induced antiviral state specially affects viral particle production, while having a lesser effect on viral genome replication. In this way, the virus may switch between a 'particle-forming' mode in the absence of IFN, and a 'genome-maintenance mode' in the presence of IFN. In the quiescent 'genome-maintenance' mode, the structured 5'UTR of V means that the level of this protein will be sufficient to dismantle the antiviral state by targeting STAT1 for degradation, a process which requires only a few hundred copies of V⁸. Once STAT1 has been degraded and IFIT1 is no longer present at a high concentration, M translation will no longer be inhibited and viral particle production will resume.

The maintenance of the viral genome concomitant with suppression of viral particle production would constitute an effective means of establishing a persistent infection. A reduction in antigen presentation and CPE would reduce recognition of viral antigens by the adaptive immune system and prevent the clearance of infected cells by antibody-dependent cellular cytotoxicity (ADCC) and cytotoxic T cells. Interestingly, the immunodominant T cell response during PIV5 infection is directed against an epitope in the M protein³⁵⁹, the translation of which is greatly reduced by IFIT1/IFN. The dependence of this proposed mechanism of persistence on post-transcriptional gene regulation mediated by IFIT1 would require chronic induction of IFIT1 by IFN^{360,361} to maintain pseudo-latency. The V protein of PIV5 efficiently disrupts IFN signalling by targeting STAT1 for degradation⁷, but an isolate of PIV5 from a persistently-infected dog⁸² encodes a defective V protein that cannot bind STAT2⁸⁴, suggesting that an increased sensitivity to IFN is able to promote persistent infection. Alternatively, infection of cells which have high basal levels of either IFIT1 or IFN would promote persistence of PIV5 without the need to induce IFN.

Many bat cells lines have been shown to constitutively express IFN/ISGs even in the absence of infection^{362–365} and it would be interesting to investigate the many rubulaviruses for which bats are the natural host. More relevant to PIV5 would be specific tissues in primates in which either IFN or ISGs are expressed at high basal levels. For example, stem cells have been shown to express high levels of ISGs even in the absence of IFN³⁶⁶; a human bronchial epithelial cell line has high basal expression of IFN and ISGs^{367,368}; keratinocytes constitutively express IFN- κ ^{369,370}; and neurons express elevated levels of IRF3 and IRF7³⁷¹. Expression of IFIT1 itself is elevated in the Müller glial cells of the retina³⁷². Immune privileged sites are often locations of

RNA virus persistence, for example the eyes³⁷³, CNS³⁷⁴, and testes³⁷⁵. Interestingly, the highly cytopathic EBOV can persist in the eyes and testes of survivors for many years in the absence of symptoms^{376,377}. Sequencing of RNA from these individuals reveals a low level of divergence from the sequence of the initial infection^{378,379}, suggesting that the rate of RNA replication is very low and that mutations are not required for the establishment of pseudo-latency by EBOV in the eye. The activity of cytotoxic T cells is highly regulated in immune privileged sites, explaining the failure to clear the virus, but this does not explain why the virus is not inducing cell death as it does in other cells³⁸⁰, nor why the replication of the virus is apparently repressed. An explanation could be that certain cells in immune privileged sites are in some form of perpetual anti-viral state that is able to restrict viral replication but not clear the RNA genome from the cells. If this antiviral state has a differential effect on the structural and non-structural genes of EBOV, a persistent infection may be established in a manner analogous to the proposed mechanism of IFIT1 specifically inhibiting the translation of PIV5 M. In this model, the acute infection would serve to disseminate the virus throughout the body and 'seed' immune privileged sites. Once adaptive immunity has been acquired, the virus is cleared from most tissues but the genome remains present in certain immune privileged sites in a pseudo-latent state. Once the adaptive immune response wanes, either due to transient immunosuppression or simply the passing of time, the production of a small number of viral particles would be sufficient to initiate a 'new' infection and potentially spread to a new host.

The various ways in which paramyxoviruses establish persistent infection can be divided into three categories. The acquisition of multiple mutations in M that permit the persistence of MV in the CNS can be referred to as 'irreversible/suicidal' persistence because the extent of the mutations in M mean that particle production cannot be restored. This mechanism is unlikely to be of evolutionary benefit to the virus because it cannot spread from the brain to new hosts. A second category of persistence can be termed 'reversible/acquired' and is exemplified by changes in the PIV5 P protein which alter the level of polymerase activity. Because these changes are encoded by codons which differ by only one nucleotide, the persistence phenotype can be easily reversed. In contrast to the acquisition of changes in P that facilitate persistence, IFIT1-mediated post-transcriptional gene regulation depends only upon induction of IFN and can therefore be termed 'reversible/inherent'; no viral mutations are required and the persistent state can be exited simply due to an absence of IFIT1.

Finally, the differential sensitivity of PIV5 genes to IFIT1 raises the possibility that other viruses have also evolved to be partially sensitive to ISGs. The antiviral activity of many ISGs is easily overcome by viruses by simply avoiding the generation of 'non-self' structures such as cap0 mRNA, dsRNA, and CpG motifs. Although the cumulative impact of having to, for example,

generate cap1 mRNA and avoid genomic CpG motifs may serve to limit the replicative rate of viruses, the failure of any virus to successfully evade an ISG should raise questions of whether this sensitivity could have been selected for in some way. For example, the E (envelope protein) ORF of SARS-CoV-2 has an elevated CpG content relative to other ORFs³⁸¹. Similar to the mechanisms proposed here, Digard *et al.* speculate that the ISG ZAP, which targets CpG motifs, could mediate specific degradation of the E mRNA as a form of translational regulation. Furthermore, Rubella virus has a CpG content that is not only not suppressed, but is increased relative to what would be expected in the absence of any selective pressure¹³¹. The potential 'deliberate' sensitivity of viruses to ISGs could preclude the inference of viral host tropism from the composition of their genomes, as has been attempted by correlating the CpG content of viral genomes to the ISGs of their hosts^{382,383} (based on the assumption that ISG mRNAs are expressed during the antiviral state and must thus be resistant to ISG proteins such as ZAP³⁸⁴). This approach may be undermined by apparently maladapted viruses which are actually able to cause persistent infections in an ISG-dependent manner.

The proposals made here are highly speculative and lack any firm supporting data. However, the extensive circumstantial evidence cited suggests that the role of IFN and ISGs in viral infections may not be as simple as commonly thought. For PIV5, more sequencing data is needed from in vivo infections to identify tissues in which this virus establishes persistent infection. The characterisation of cells in immune privileged sites will also be necessary to determine how RNA virus replication is repressed in a way which fails to clear the viral genome. The continued emergence of zoonotic RNA viruses with varying host tropism and IFN sensitivity makes the persistent infection of immune privileged sites an ongoing concern.

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