

Functional Analysis of the African Swine Fever Virus MGF360 and MGF505 Proteins, and its Application to Vaccine Development



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DECLARATION

I declare that this thesis is entirely my own work, and except where otherwise stated, describes my own research.

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ABBREVIATIONS

2'-5' OAS	2'-5'oligoadenylate synthetase
3AT	3-Amino-1,2,4-triazole
βgal	βgalactosidase
Ade	Adenine
APOBEC	Apolipoprotein B mRNA editing enzyme catalytic polypeptide
ASF	African swine fever
ASFV	African swine fever virus
BAF	Brahma related gene Brahma associated factor
BSA	Bovine serum albumin
CARD	Caspase activating and recruiting domain
CBP	CREB binding protein
cGAS	Cyclic GMP-AMP synthase
CoIP	Co-Immunoprecipitation
CREB	cAMP-response element binding protein
CRL	Cullin-RING-E3 Ubiquitin Ligase
CSF1-fc	Macrophage colony-stimulating factor fused to the Fc of porcine IgG1a
Cul	Cullin
CXCL10	C-X-C motif chemokine ligand 10
CXCR3	C-X-C motif receptor 3
DAPI	4',6-diamidino-2-phenylindole
DBD	DNA binding domain
DMEM	Dulbecco's modified Eagle medium

DMSO	Dimethyl sulphoxide
dsDNA	Double stranded DNA
dsRNA	Double stranded RNA
DTT	Dithiothreitol
EBSS	Earle's balanced salt solution
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immune absorbent spot assay
EloA	Elongin A
EloB	Elongin B
EloC	Elongin C
EMSA	Electrophoretic mobility shift assay
FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum
Gal4DBD	Gal4 DNA binding domain
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HIF- α	Hypoxia-inducible factor α
His	Histidine
IAD	IRF association domain
IFN	Interferon
IFNAR	IFN $\alpha\beta$ receptor
IKK	I κ B kinase
IL1R	Interleukin 1 β receptor
IL-1 β	Interleukin 1 β

IM	Intramuscularly
IRAK	Interleukin-1 receptor-associated kinase
IRF	Interferon regulatory factor
ISG	IFN stimulated gene
ISGF3	ISG factor 3
ISRE	IFN-stimulated response element
JAK	Janus kinase
KIX	Kinase-inducible domain interacting domain
LAV	Live attenuated vaccine
Leu	Leucine
LGP2	Laboratory of genetics and physiology 2
LOD	Limit of detection
MAVS	Mitochondrial antiviral-signaling protein
MDA5	Melanoma differentiation-associated protein 5
MGF	Multigene family
MVA	Modified vaccina Ankara
MX	Myxoma resistance protein
NAP1	NFκB activating kinase - associated protein 1
NCLDV	Nucleocytoplasmic large DNA viruses
NFκB	Nuclear factor κB
OD600	Optical density of 600nm
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PEG	Polyethylene glycol
PIC	Pre-Initiation complex
PKR	Protein kinase R
PRD	Positive regulatory domain
PRR	Pathogen recognition receptor
PS	Penicillin streptomycin
PVDF	Polyvinylidene difluoride
qPCR	Quantitative PCR
RIG-I	Retinoic acid inducible gene-I
RIP1	Receptor interacting protein 1
RIPA	Radioimmunoprecipitation assay buffer
RLR	RIG-like receptor
RNAPII	RNA polymerase II
Rpb1	RNAPII subunit B1
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute Media
RT	Room temperature
SAPO4	Specified animal pathogen order
SDCBP	Syndecan-binding protein
SDS	Sodium dodecyl Sulphate
SERTAD3	SERTA Domain 3
SINTBAD	Similar to NAP1 TBK1 adaptor protein
SOCS	Suppressor of cytokines
ssRNA	Single stranded RNA
SILAC	Stable isotope labelling by amino acids in cell culture

STAT	Signal transducers and activators of transcription
STING	Stimulator of interferon genes
TAB	TAK1-binding protein
TAD	Transactivation domain
TAK1	TGF β activated kinase 1
TANK	TRAF family member associated NF κ B activator
TBK1	Tank binding kinase 1
TBST	TRIS-buffered saline tween
TE	TRIS-EDTA buffer
TGF β	Transforming growth factor β
TGS	TRIS-glycine SDS
TIR	Toll IL1 receptor
TLR	Toll-like receptor
TNFR	TNF α receptor
TNF α	Tumour necrosis factor α
TRAF	TNF α receptor associated factor
TRIF	TIR-domain-containing adapter-inducing interferon- β
TRIM25	Tripartite motif containing protein 25
Trp	Tryptophan
Tyk2	Tyrosine kinase 2
UAS	Upstream activation sequence
Ub	Ubiquitin
VHL	Von Hippel Lindau
YPD	Yeast extract peptone dextrose
YPDA	Yeast extract peptone dextrose adenine

CONTENTS

DECLARATION	i
ACKNOWLEDGMENTS	ii
ABBREVIATIONS	iv
CONTENTS.....	ix
FIGURES.....	xvi
TABLES.....	xix
ABSTRACT	xx
1 INTRODUCTION.....	1
1.1 Immune Response to Viruses.....	1
1.1.1 The Innate Immune Response.....	2
1.1.2 Type-I Interferons	3
1.1.3 Induction of Type-I Interferon Expression	4
1.1.4 Type-I IFN Stimulating Transcription Factors.....	5
1.1.4.1 Interferon Regulatory Factors.....	5
1.1.4.2 Nuclear Factor κ B.....	7
1.1.5 Recognition of Viral Pathogens.....	8
1.1.5.1 Transmembrane Pathogen Recognition Receptors.....	8
1.1.5.2 Cytosolic Pathogen Recognition Receptors	10
1.1.6 Interferon Stimulated Genes.....	16
1.1.7 Viral Antagonism of the Type-I IFN Response.....	18
1.1.7.1 1 - Subversion of IFN Induction Cascades.....	19
1.1.7.2 2 - Subversion of IFN Signalling Pathway	21
1.1.7.3 3 - Blockage of Antiviral ISG Products	21
1.1.7.4 Utilization of Cullin-RING-Ligase Machinery by IFN Pathway Modulators.....	22
1.2 African Swine Fever	28

1.2.1	Epidemiology	28
1.2.2	Pathology	31
1.2.3	Virology	32
1.2.3.1	Viral Structure	32
1.2.3.2	ASFV Genomics	34
1.2.3.3	ASFV Replication Cycle	35
1.2.4	Multigene Family Genes	39
1.2.5	Other Immunomodulatory ASFV Genes of Note	43
1.2.6	ASFV Vaccinology	45
1.2.6.1	Inactivated Vaccines	46
1.2.6.2	Subunit and Virus Vectored Vaccines	47
1.2.6.2.1	Antigen Vaccines	48
1.2.6.2.2	Viral Vectored Vaccines	48
1.2.6.3	Live Attenuated Vaccines	50
1.2.7	Aims of the Project	54
2	MATERIALS & METHODS	55
2.1	Computational Bioinformatics Analysis	55
2.2	Cell Culture	56
2.2.1	Cell Lines	56
2.2.2	Isolation and Differentiation of Primary Cells	56
2.3	Virus Methods	57
2.3.1	Propagation of Viral Stocks	57
2.3.2	TCID ₅₀ Viral Titration	58
2.4	Plasmids and Nucleic Acid Techniques	59
2.4.1	RNA Extraction	59
2.4.2	Viral DNA Extraction	59
2.4.3	cDNA Synthesis	60
2.4.4	Plasmids and Cloning	60
2.4.5	Vectors	60
2.4.6	General Cloning Techniques	62
2.4.6.1	Polymerase Chain Reaction Amplification of Target Inserts	62

2.4.6.2	Agarose Gel Electrophoresis	64
2.4.6.3	Restriction Enzyme Digestion of DNA.....	64
2.4.6.4	Ligations	65
2.4.7	Site Directed Mutagenesis	65
2.4.8	Transformation Of <i>E. Coli</i> and Plasmid Scale Up	66
2.4.9	Sanger Sequencing	67
2.5	Yeast-2-Hybrid.....	69
2.5.1	Transfection of Yeast Cells	69
2.5.2	Selection of Optimal Growth Conditions for Yeast	70
2.5.3	Mating of Transfected Yeast Strains	71
2.5.4	Yeast-2-Hybrid Interaction Assays	71
2.6	Luciferase Assays	72
2.6.1	Luciferase Assay - Cell Preparation	72
2.6.2	Luciferase Assay – Transfection	72
2.6.3	Luciferase Assay – Stimulation of Cells.....	73
2.6.4	Dual Luciferase Assay – Cell Lysis and Luciferase Reading.....	73
2.6.5	β Galactosidase Assay – Cell Lysis and Reading	74
2.7	Protein Characterisation	75
2.7.1	SDS-PAGE – Western Blotting Sample Preparation.....	75
2.7.2	SDS-PAGE – Co-Immunoprecipitation	75
2.7.2.1	Antibody Coupling to Magnetic Epoxy M-270 Beads.....	75
2.7.2.2	CoIP – Sample Preparation	76
2.7.3	SDS-PAGE – Gel Electrophoresis	77
2.7.4	Protein Transfer to PVDF Membranes.....	77
2.7.5	Membrane Blotting.....	77
2.8	Immunofluorescent Microscopy	80
2.8.1	Immunofluorescent Staining	80
2.9	qPCR Analysis.....	82
2.9.1	Infection of Porcine Bone Marrow Cells for qPCR Gene Expression Analysis	82
2.9.2	Removal from SAP04.....	82

2.9.3	qPCR.....	83
2.10	Statistical Analysis	86
3	BIOINFORMATIC ANALYSIS OF THE MGF360 & MGF505 GENES	87
3.1	Introduction.....	87
3.2	Results.....	88
3.2.1	BLASTp Provided no Proteins of Significant Homology but Pointed Towards the Presence of Ankyrin (ANK) Repeats	88
3.2.2	Putative Structural Analysis and Motif Homology Indicates the Presence of Ankyrin Repeats	90
3.2.3	Both MGF360 & MGF505 Cluster Proteins have a Putative VHL/SOCS-Box-Like Motif	96
3.2.4	Predicted Structure of the BC-Box & Cul2/5-Box of the MGFs Show Similarity to that of Cellular Counterparts	105
3.2.5	MGF360-11L, 12L & 14L have a Predicted TRAF6 Binding Motif	107
3.2.6	MGF505-2R has a Predicted IRF3 Binding Motif	109
3.3	Discussion.....	110
4	YEAST-2-HYBRID ANALYSIS PROBING FOR INTERACTING PARTNERS OF THE MGF360 & MGF505 PROTEINS.....	118
4.1	Introduction.....	118
4.2	Results.....	119
4.2.1	Yeast-2-Hybrid Assay Overview.....	119
4.2.2	MGF Constructs were Tested for Self-Activation.....	124
4.2.3	MGF360-11L, 12L, 13L & MGF505-3R Interacted with TRAF6.....	124
4.2.4	MGF360-12L Interacted with TRAF3	126
4.2.5	MGF360-13L Interacted with IRF3.....	127
4.3	Discussion.....	128
5	INHIBITION OF THE NFκB SIGNALLING PATHWAY BY MGF360 & MGF505 PROTEINS	138
5.1	Introduction.....	138

5.2	Results.....	143
5.2.1	MGF360 & MGF505 Inhibited the NF κ B Pathway at the Transcription Factor Level or Below	143
5.2.2	MGF360-12L & MGF505-1R Inhibit the NF κ B Pathway in a Dose Dependant Manner.....	147
5.2.3	MGF360-11L & MGF505-2R Inhibited p65 Nuclear Translocation 149	
5.2.4	MGF360-12L & MGF505-1R did not Impact the Expression of Selected Components of the NF κ B Pathway	154
5.2.5	Impact of MGF360-12L's Putative Novel TRAF6 Binding Site on Inhibition of the NF κ B Pathway	156
5.2.6	MGF360-12L did not Co-Immunoprecipitate with Endogenous TRAF6 or p65.....	160
5.2.7	MGF505-1R did not Co-Immunoprecipitate with Endogenous p65 162	
5.2.8	MGF360-12L Inhibits p65 Transcriptional Activity in a Luciferase Assay Under the Control of the GAL4 promoter.....	164
5.3	Discussion.....	168

6	INHIBITION OF THE IRF3 SIGNALLING PATHWAY BY MGF360 & MGF505 PROTEINS	175
6.1	Introduction.....	175
6.2	Results.....	179
6.2.1	MGF360 & MGF505 Inhibited the IRF3 Pathway at the Transcription Factor Level or Below.....	179
6.2.2	MGF360-12L Partially Inhibits Nuclear Translocation of IRF3	183
6.2.3	Impact of MGF360-12L & MGF505-1R on Key Components of the IRF3 Pathway.....	188
6.2.4	MGF360-12L did not Co-Immunoprecipitate with Endogenous IRF3 190	
6.2.5	MGF360-12L Inhibits IRF3 Transcriptional Activity in a Luciferase Assay Under the Control of the GAL4 Promoter	192

6.3	Discussion.....	194
7	UNDERSTANDING THE MECHANISM(S) OF ACTION OF MGF360-12L & MGF505-1R DRIVEN INHIBITION OF THE NFκB AND IRF3 PATHWAYS.....	201
7.1	Introduction.....	201
7.2	Results.....	206
7.2.1	Generation of MGF360-12L & MGF505-1R Mutants	206
7.2.2	MGF360-12L Co-Immunoprecipitates with Endogenous VHL CRL Machinery	208
7.2.3	MGF05-1R Co-Immunoprecipitates with SOCS CRL Machinery..	212
	Mutation of	217
7.2.4	MGF505-1R SOCS-Box Alleviated Inhibition of the NF κ B & IRF3 Pathways, but Mutation of MGF360-12L VHL-Box did not.....	217
7.2.5	MGF360-12L Reduced the Expression of Specific Transfected Plasmids	220
7.2.6	MGF505-1R's SOCS-Box Motif is Required for a Reduction in Endogenous Levels of p300	224
7.2.7	Neither MGF360-12L or MGF505-1R Impacted upon TFIIIB or Rpb1 Endogenous Protein Levels	228
7.3	Discussion	231
8	DELETION OF MGF360-12L, 13L, 14L, MGF505-1R & K145R FROM VIRULENT GEORGIA07/1 ALTERS ISG TRANSCRIPT LEVELS AND CONFERS PROTECTION AGAINST CHALLENGE WITH PARENTAL VIRUS	243
8.1	Introduction.....	243
8.2	Results.....	246
8.2.1	Selection of a Profile of MGFs for Deletion from Georgia07/1 ...	246
8.2.2	Georgia Δ MGFAK145R Showed an Impaired Ability to Inhibit the Expression of IFN Stimulated Genes	252
8.2.3	In Vivo Challenge Study of Vaccine Candidates Showed that Georgia Δ MGFA Δ K145R Induced Protection Against Challenge with Georgia07/1, whereas Georgia Δ MGFB Δ K145R was not Attenuated.....	255

8.2.4	Vaccination with Georgia Δ MGFA Δ K145R did not Provide Sterile Immunity	261
8.3	Discussion	264
9	DISCUSSION.....	271
10	REFERENCES.....	283
11	SUPPLEMENTARY FIGURES	321

FIGURES

<i>Figure 1-1. Schematic showing an overview of the induction of the IRF3 pathway</i>	<i>13</i>
<i>Figure 1-2. Schematic giving overview of NFκB pathway activation</i>	<i>15</i>
<i>Figure 1-3. Structure of a substrate binding protein bound to the CRL machinery and substrate</i>	<i>27</i>
<i>Figure 1-4. Global map of ASFV presence.....</i>	<i>30</i>
<i>Figure 1-5. Overview of ASFV structure.....</i>	<i>33</i>
<i>Figure 1-6. Overview of the ASFV replication cycle</i>	<i>38</i>
<i>Figure 1-7. Architecture of Georgia07/1 genome</i>	<i>44</i>
<i>Figure 3-1. MGF360-14L SWISS-MODEL built against DARPIn variant DDD_D12_06_D12_06_D12 (5leb.1.A)</i>	<i>93</i>
<i>Figure 3-2. Alignment MGF360 and MGF505 proteins with various VHL-Box and SOCS-Box proteins.</i>	<i>98</i>
<i>Figure 3-3 Comparison of MGF505 BC-Box motif to that of known VHL-Box and SOCS-Box proteins</i>	<i>100</i>
<i>Figure 3-4. Alignment of MGF360 proteins against sequences of known Cul-Box motifs.....</i>	<i>102</i>
<i>Figure 3-5 Alignment of MGF505 proteins against sequences of known Cul-Box motifs.....</i>	<i>103</i>
<i>Figure 3-6. Alignment of MGF360 and MGF505 proteins highlighting the VHL-Box and SOCS-Box consensus sequence.</i>	<i>104</i>
<i>Figure 3-7 . Jpred analysis showing the predicted secondary structure of MGF360 and MGF505 proteins</i>	<i>105</i>
<i>Figure 3-8. TRAF6 binding motifs in MGF360-11L, 12L and 14L.....</i>	<i>108</i>
<i>Figure 3-9. IRF3 binding motif of MGF505-2R.....</i>	<i>109</i>
<i>Figure 3-10 Schematic showing proposed architecture of the proteins analysed in MGF360 and MGF505 clusters</i>	<i>117</i>
<i>Figure 4-1. Overview of the critical stages of the yeast-2-hybrid assay</i>	<i>122</i>
<i>Figure 4-2. Key principle of the yeast-2-hybrid assay</i>	<i>123</i>
<i>Figure 4-3. Interactors of TRAF6.....</i>	<i>125</i>
<i>Figure 4-4. MGF360-12L interacts with TRAF3.....</i>	<i>126</i>
<i>Figure 4-5. MGF360-13L interacts with IRF3</i>	<i>127</i>

<i>Figure 4-6. The role of TRAF3 in the signalling pathways leading to IFN expression</i>	<i>131</i>
<i>Figure 4-7. The role of TRAF6 in the signalling pathways leading to IFN expression</i>	<i>132</i>
<i>Figure 5-1. Schematic giving simple overview of activation of the NFκB pathway</i>	<i>139</i>
<i>Figure 5-2. Luciferase data showing assessment of the MGFs inhibition of the NFκB pathway</i>	<i>146</i>
<i>Figure 5-3. Dose dependent inhibition of the NFκB pathway by MGF360-12L and MGF505-1R.....</i>	<i>148</i>
<i>Figure 5-4. Impact of MGFs on nuclear translocation of p65</i>	<i>153</i>
<i>Figure 5-5. MGF360-12L and MGF505-1R do not impact on NFκB pathway functionality</i>	<i>155</i>
<i>Figure 5-6. Impact of mutation of the putative novel TRAF6 binding motif in MGF360-12L on inhibition of the NFκB pathway</i>	<i>158</i>
<i>Figure 5-7. MGF360-12L did not co-immunoprecipitate with endogenous p65 or TRAF6</i>	<i>161</i>
<i>Figure 5-8 MGF505-1R did not co-immunoprecipitate with endogenous p65</i>	<i>163</i>
<i>Figure 5-9. Analysis of MGFs on the transcription activity of Gal4DBD-p65</i>	<i>165</i>
<i>Figure 5-10. Schematic giving overview and explanation of data observed from luciferase assays.</i>	<i>167</i>
<i>Figure 6-1. Schematic showing simple overview of activation of the IRF3 pathway.....</i>	<i>176</i>
<i>Figure 6-2. Luciferase assay investigating inhibition of the IRF3 pathway by MGF360 and MGF505 proteins</i>	<i>181</i>
<i>Figure 6-3. MGF360-12L impacted on the nuclear translocation of IRF3.....</i>	<i>186</i>
<i>Figure 6-4. MGF360-12L and MGF505-1R did not appear to impact IRF3 protein levels.....</i>	<i>189</i>
<i>Figure 6-5. CoIP probing for an interaction between MGF360-12L.V5 and endogenous IRF3</i>	<i>191</i>
<i>Figure 6-6. Impact of MGFs on transcriptional activity of IRF3.....</i>	<i>193</i>
<i>Figure 7-1. Overview of the stages of de novo transcription initiation of the IFNβ gene</i>	<i>205</i>
<i>Figure 7-2. Mutations of the putative VHL/SOCS-Boxes of MGF360-12L and MGF505-1R</i>	<i>207</i>
<i>Figure 7-3. CoIP probing for interacting partners of MGF360-12L</i>	<i>210</i>
<i>Figure 7-4. CoIP probing for interacting partners of MGF505-1R</i>	<i>214</i>
<i>Figure 7-5. Mutations in the SOCS-Box of MGF505-1R alleviate inhibition of the IRF3 and NFκB pathways</i>	<i>219</i>
<i>Figure 7-6. Impact of MGF360-12L on expression of exogenous plasmids</i>	<i>222</i>
<i>Figure 7-7. MGF505-1R impacts upon the level of endogenous p300.....</i>	<i>226</i>
<i>Figure 7-8. CoIP probing for interacting partners of MGF360-12L.....</i>	<i>229</i>

<i>Figure 8-1. Schematic depicting the generation of deletion mutants lacking member of MGF360 and MGF505 genes</i>	<i>251</i>
<i>Figure 8-2. qPCR analysis of ISG expression at 0, 2, 8, 16, and 24 hpi.....</i>	<i>254</i>
<i>Figure 8-3. Overview of in vivo challenge study</i>	<i>256</i>
<i>Figure 8-4. Survival rates of Group A, B and C.....</i>	<i>257</i>
<i>Figure 8-5. Temperature scoring of individual animals throughout the study.....</i>	<i>259</i>
<i>Figure 8-6. Clinical scoring for individual animals per day throughout the study</i>	<i>260</i>
<i>Figure 8-7. qPCR determined viral genome copies in blood samples</i>	<i>263</i>

TABLES

<i>Table 2-1. Software used in computational analysis</i>	<i>55</i>
<i>Table 2-2. Primer sequences used for gene amplification for cloning</i>	<i>63</i>
<i>Table 2-3. PCR conditions</i>	<i>68</i>
<i>Table 2-4. Antibodies used for western blotting and CoIP</i>	<i>79</i>
<i>Table 2-5. Antibodies used in immunofluorescent staining.....</i>	<i>81</i>
<i>Table 2-6. qPCR Programme</i>	<i>84</i>
<i>Table 2-7. Primers used for qPCR analysis.....</i>	<i>85</i>
<i>Table 3-1. Hmmscan predicted presence of ANK repeats within MGFs</i>	<i>95</i>
<i>Table 4-1. Yeast-2-Hybrid “Bait” showing insert and vector</i>	<i>120</i>
<i>Table 4-2 Yeast-2-Hybrid “Prey” showing insert and vector.....</i>	<i>120</i>
<i>Table 4-3. Overview of the potential interactions between MGFs and components of the IFN signalling pathway</i>	<i>128</i>
<i>Table 5-1. Overview of the results of the impact of the MGFs on the NFκB signalling pathway.....</i>	<i>169</i>
<i>Table 6-1. Overview of the results of the impact of the MGFs on the IRF3 signalling pathway.....</i>	<i>195</i>
<i>Table 7-1. Overview of the observed interacting partners of the VHL/SOC-Box CRL machinery with MGF360-12L, and MGF505-1R.....</i>	<i>216</i>
<i>Table 7-2 Typical values of unstimulated luciferase assay readings</i>	<i>223</i>
<i>Table 7-3. Overview of results investigating role of the VHL/SOCS box in the functionality of MGF360-12L and MGF505-1R.</i>	<i>232</i>
<i>Table 11-1 Alignments of BLASTp hits for MGFs of interest.....</i>	<i>322</i>

ABSTRACT

African swine fever virus (ASFV) is a deadly haemorrhagic pathogen of swine. In recent years, ASFV has spread beyond Africa and continues to ravage European and Asian pig herds, posing a severe socio-economic threat. No vaccines are currently available leaving preventive and extermination measures as the only course of action. The virus' dsDNA genome is up to 190kb in length, and contains 5 clusters of paralogous genes known as Multigene family (MGF) genes close to both termini. Genes of MGF360 and MGF505 clusters have been implicated in the subversion of the innate immune system, and their absence is known to attenuate virulent isolates, however their specific mechanism of action is unknown. Here I have characterised the functions of MGF360-10L, 11L, 12L, 13L, 14L, MGF505-1R, 2R and 3R, by performing *in silico* and *in vitro* analysis. I revealed that these MGF genes inhibit the interferon signalling pathways at the level of the transcription factors or below. MGF360-12L and MGF505-1R were demonstrated to both contain a functional novel Cullin-RING-E3 ubiquitin ligase (CRL) recruitment motif, which was indispensable for the inhibitory action of MGF505-1R on the interferon pathway. Moreover MGF505-1R reduced levels of endogenous p300, a cofactor essential in the transcription of type-I Interferon and other genes. Mutagenesis of the CRL motif restored p300 levels, indicating that MGF505-1R targets the p300 cofactor for CRL driven ubiquitination and subsequent proteasomal degradation. I applied the knowledge gained here to the rational development of vaccine candidates. Two profiles of MGF genes were deleted from the virulent Georgia07/1, and the impact on *ex vivo* ISG expression,

and *in vivo* attenuation in pigs was assessed. This revealed the specific genes potentially key for attenuation. Overall, I have advanced knowledge on understanding the molecular mechanisms of the ASFV MGF360 and MGF505 proteins and identified two of those with critical roles during virus infection.

**Functional Analysis of the African Swine Fever Virus
MGF360 and MGF505 Proteins, and its Application to
Vaccine Development**

1 INTRODUCTION

The body of this thesis will focus on African swine fever (ASF) virus (ASFV) multigene family (MGF) genes belonging to clusters MGF360 and MGF505. Although these are known to be inhibitors of the innate immune system, their mechanism of action remains undetermined. Here I aim to functionally characterise the mechanism of action of individual MGF genes, and to apply this knowledge to vaccine development. I therefore will begin with an introduction into the innate immune system and its interplay with viral pathogens before discussing ASFV. I conclude with how the MGF genes are relevant to the virus' immune evasion strategy, and their significance to the development of live attenuated (LAV) vaccines.

1.1 Immune Response to Viruses

The interplay between viruses and their hosts has been ongoing for millions of years. This has in essence resulted in an evolutionary arms race between the two. Viruses have evolved numerous elegant strategies to hijack their hosts' molecular machinery repurposing it for their own advantage, whereas the host has developed an armoury of defence strategies to limit viral replication (Akira, Uematsu *et al.* 2006, Randall and Goodbourn 2008, Goubau, Deddouche *et al.* 2013, Hertzog and Rehwinkel 2020). In gnathostomes (jawed vertebrates) this has evolved into a highly complex set of interconnected pathways which can be divided into two primary arms of defence: the innate, and the adaptive immune response. The innate response is found across all cellular life and is responsible

for mounting the first response to infection. By deploying germline encoded sentinels that recognise highly specific pathogen determinants, cells are able to initiate rapid changes within the cellular environment to combat the infection. The innate immune response does not, however, provide a uniquely tailored response to a specific pathogen, this falls within the remit of the adaptive response. Crucially both arms function synergistically, initiation of the innate response leads to activation of the adaptive (Iwasaki and Medzhitov 2015). In contrast to the innate response, the adaptive response is only found in higher order organisms, and builds a broad and highly specific repertoire against an almost infinite number of targets. Furthermore, this response develops into immunological memory allowing the host to mount a rapid and exceptionally precise response to individual pathogens on the second exposure. The highly coordinated efforts of these two systems in synergy allows the host to be able to mount an appropriate immune response to the viral invader.

1.1.1 The Innate Immune Response

Infection by pathogens poses an obvious challenge for the host to overcome. Cellular life has evolved a multitude of differing mechanisms in order to deal with viral invaders. The common theme of which is the recognition of conserved motifs unique to pathogens, known as pathogen associated molecular patterns (PAMP)s (Janeway 1992). In the context of viral infection these motifs are various forms of nucleic acids including single stranded RNA (ssRNA), double stranded RNA (dsRNA), viral DNA, or viral proteins. The cellular machinery which recognises these PAMPs include several classes of germline encoded receptors,

known as pathogen recognition receptors (PRR)s (Akira, Uematsu *et al.* 2006, Randall and Goodbourn 2008, Goubau, Deddouche *et al.* 2013, Hertzog and Rehwinkel 2020). Prokaryotic life employs endonucleases, whereas eukaryotic life has evolved an intricate and complex set of PRRs and downstream signalling pathways to induce the first line of defence against pathogens. In the context of viral infection PRRs can be broadly divided into those that are transmembrane, or cytosolic. The recognition of PAMPs by PRRs stimulates the innate immune system by activating a series of downstream pathways that ultimately culminate in the activation of transcription factors and induce the expression of type-I interferon (IFN) and other inflammatory cytokines (Akira, Uematsu *et al.* 2006, Honda and Taniguchi 2006, Randall and Goodbourn 2008). This activation can be further divided into two primary pathways, the IFN regulator factor (IRF), and Nuclear Factor κ B (NF κ B) pathways. IFN acts as a signalling protein that functions in both an autocrine and paracrine manner. Its production leads to altered expression of over 300 different genes, known as IFN stimulated genes (ISG)s, creating an antiviral environment in order to limit the replication of the invading viral pathogen (Der, Zhou *et al.* 1998, Granja, Nogal *et al.* 2006, Sadler and Williams 2008, Granja, Sánchez *et al.* 2009). There is a strong selection for viruses to impede this innate immune response. The evolutionary arms race between virus and host has resulted in the vast majority of, if not all, viruses that infect metazoans deploying tactics to overcome and subvert these pathways.

1.1.2 Type-I Interferons

Response to viral infection by the innate immune system ultimately leads to the production of type-I interferons. This is a class of cytokines that include 13

variants of IFN α in humans, a single IFN β , and a number of poorly defined but related cytokines such as IFN κ and IFN δ (McNab, Mayer-Barber *et al.* 2015). Interestingly swine however have 39 functional type-I interferons, including 17 variants of IFN α (Sang, Rowland *et al.* 2010, Zanotti, Razzuoli *et al.* 2015). These polypeptides are secreted by the cell and can act in an autocrine or paracrine fashion by binding to the dimeric IFN $\alpha\beta$ receptor (IFNAR). This activates downstream pathways leading to the development of the antiviral environment.

1.1.3 Induction of Type-I Interferon Expression

Almost all cells are capable of inducing type-I IFN β following viral infection, whereas IFN α is primarily produced by dendritic cells. Induction of IFN β expression occurs in a highly ordered process at the transcriptional level by activation of the IFN β promoter. This promoter can be divided into at least 4 cis regulatory element regions known as positive regulatory domains (PRD) I, II, III and IV (Kim and Maniatis 1997). When activated by upstream signalling pathways transcription factors of the IRF family bind to PRDI/III, whereas NF κ B and AP1 (cJUN, ATF2 heterodimer) bind to regions PRDII or PRDIV respectively (See 1.1.4). The binding of the transcription factors results in the formation of a higher order nucleoprotein complex termed the enhanceosome. Further recruitment of histone acetyl transferase cofactor proteins including cAMP-response element binding protein (CREB) binding protein (CBP), or paralog p300 is required (Merika, Williams *et al.* 1998). These cofactors aggregate at the promoter to target H3 and H4 histones of the nucleosome for acetylation. This relaxes the chromatin structure at the promoter, providing a platform for the

cellular machinery to facilitate transcription (Gerritsen, Williams *et al.* 1997, Merika, Williams *et al.* 1998, Yoneyama, Suhara *et al.* 1998, Qin, Liu *et al.* 2005, Mukherjee, Behar *et al.* 2013). Transcription is further dependent upon a number of additional proteins including the RNA polymerase II (RNAPII) holo complex, and at least 6 general transcription factors TFIIA, TFIIB, TFIID, TFII E, TFII F, and TFII H. These together form the preinitiation complex (PIC) to induce transcription. CBP/p300 recruits TFIIB to the promoter allowing further recruitment of TFIID. The recruitment of Brahma related gene Brahma associated factor (BAF) forces the dissociation of the chromatin nucleosome, enabling access to the TATA box of the promoter by TFIID. The recruitment of the remaining PIC is dependent on the presence of CBP/p300 at the promoter (Kim, Kim *et al.* 1998, Agalioti, Lomvardas *et al.* 2000, Honda, Takaoka *et al.* 2006).

1.1.4 Type-I IFN Stimulating Transcription Factors

As the IFN β promoter is under the control of a number of cis acting regulatory elements this allows expression to be driven by various transcription factors, IRFs, NF κ B, and AP1 (cJUN ATF2 heterodimer) (Honda, Takaoka *et al.* 2006). In the context of viral infection, IRF3 and NF κ B play a major role in the induction of IFN β expression. I will therefore focus upon these two transcription factors.

1.1.4.1 Interferon Regulatory Factors

The interferon regulatory factor (IRF) class of transcription factors contains up to 9 conserved members, IRF1-9. The architecture of these transcription factors can be described as having an N-Terminal DNA binding domain (DBD), with a C-terminal IRF association domain (IAD) responsible for the formation of homo or

heterodimeric complexes of IRFs. Although it is of note that the IAD is absent in IRF1 and IRF2. Crystal structure analysis revealed that the DBD of IRFs forms a helix-turn-helix structure allowing the proteins to recognise specific DNA sequences of the PRDI/III IFN β promoter region (Fujii, Shimizu *et al.* 1999, Panne, Maniatis *et al.* 2004). NF κ B plays an important role in the inflammatory response and is capable of driving IFN β expression, albeit less so than IRFs. In fact, it appears that a robust IFN response to viral infection is conditional on the activation of the IRFs, specifically the constitutively expressed IRF3 (Honda, Takaoka *et al.* 2006). Inactive IRF3 remains dormant in the cytosol, until stimulation of signalling pathways following the detection of viral PAMPs leads to its activation. Phosphorylation of C-terminal regions of IRF3 by directly upstream kinases is key in the activation of the transcription factor. The closely related I κ B kinase (IKK) homologs IKK ϵ , and TANK binding kinase 1 (TBK1) induce the phosphorylation of IRF3, however TBK1 has been shown to act as the primary actor in the phosphorylation of serine residues 386, and 396 (Lin, Heylbroeck *et al.* 1998, Fitzgerald, McWhirter *et al.* 2003, Takahasi, Suzuki *et al.* 2003, Mori, Yoneyama *et al.* 2004, Panne, McWhirter *et al.* 2007, Chen, Srinath *et al.* 2008). Phosphorylation of additional residues has also been proposed to play an important role in activation, furthermore the phosphorylation of S386 and S396 are key. The phosphorylation of the S386 residue leads to the formation of the IRF3 homodimer, a critical step in activation (Takahasi, Suzuki *et al.* 2003, Mori, Yoneyama *et al.* 2004, Panne, McWhirter *et al.* 2007). This dimeric form of IRF3 subsequently translocates to the nucleus forming complexes with CBP/p300 cofactors and binds to the PRDI/III sites of the IFN promoter (Lin, Heylbroeck *et al.* 1998, Lin, Génin *et al.* 2000, Panne, McWhirter *et al.* 2007).

1.1.4.2 Nuclear Factor κ B

Although not the primary inducer of IFN β expression, NF κ B retains a crucial function in the development of an inflammatory and antiviral response. NF κ B transcription factors are a family of proteins that bind to cis acting κ B DNA elements to regulate inflammatory gene transcription. NF κ B is formed of either a homodimer, or heterodimer consisting of proteins which share the presence of a Rel domain, these include p50, p52, p65 (RelA), RelB and cRel (Li and Verma 2002, Oeckinghaus and Ghosh 2009, Israël 2010). p65, RelB and cRel contain a functional transcription activation domain, whereas the remaining members of the family do not (Oeckinghaus and Ghosh 2009). The p50 p65 heterodimer is a very common form of the NF κ B transcription factor. Here I will focus upon this p50 and p65 heterodimer due to its role in the canonical NF κ B signalling pathway, all further instances of NF κ B will refer to this heterodimer. In the canonical pathway, NF κ B can be activated by Tumour necrosis factor- α (TNF α) or interleukin 1 β (IL-1 β) stimulation of their respective receptors TNFR and IL-1 β receptor (IL1R), or PRR activation by relevant PAMPs. Inactive NF κ B is sequestered in the cytosol by the negative regulator I κ B α protein which binds to NF κ B to induce a 180° conformational change essentially locking it in a “closed” structure (Huxford, Huang *et al.* 1998). Stimulation of upstream signalling pathways results in the activation of the IKK kinase complex, which is formed of two catalytic subunits IKK α , IKK β and the regulatory subunit IKK γ . The method of activation is not clear but may occur by either autophosphorylation, or by transforming growth factor β (TGF β) activated kinase 1 (TAK1) mediated

phosphorylation of the catalytic subunits (Wang, Deng *et al.* 2001, Xu, Lo *et al.* 2011). The IKK complex then phosphorylates the negative regulator I κ B α bound to NF κ B, subsequently “unlocking” the conformation releasing the transcription factor, revealing a ubiquitination site on the negative regulator leading to its proteasomal destruction (Traenckner, Pahl *et al.* 1995, Huxford, Huang *et al.* 1998, Karin and Ben-Neriah 2000, Chen 2005). Despite the similarities between constituent catalytic proteins of the IKK complex, it has been revealed that IKK β is the principal actor in NF κ B signal transduction (Israël 2010). Activation of NF κ B eventually leads to the nuclear translocation of the transcription factor and binding to κ B elements in promoters to induce gene expression.

1.1.5 Recognition of Viral Pathogens

1.1.5.1 Transmembrane Pathogen Recognition Receptors

Transmembrane PRRs include the Toll-like receptors (TLR)s. These are a class of 13 described PRRs (TLR1-13) with a broad range of specificity (Akira and Takeda 2004, Kawasaki and Kawai 2014). TLR3/7/9 for example are located in endosomal membrane and recognise dsRNA, ssRNA, and viral DNA respectively. These recognise extracellular PAMPs generated by the uncoating of viral particles. TLRs can also recognise PAMPs of parasitic or bacterial pathogens. Activation of TLRs leads to the stimulation of downstream signalling pathways (Figure 1-1B, Figure 1-2) with the initial mediator being the intracellular Toll IL1R (TIR) domain that facilitates further signal transduction by binding adaptor proteins TIR domain-containing adapter-inducing interferon- β (TRIF) (in the case of TLR3) or MyD88 (TLR7 and TLR9). TLR3 associated TRIF interacts with the

RING-domain E3 ubiquitin (Ub) ligase TNF α receptor-associated factor (TRAF)6, but also TRAF3 (Häcker, Redecke *et al.* 2006). TRAF6 mediated signalling leads to IKK complex activation and therefore phosphoactivation of NF κ B (Wang, Deng *et al.* 2001, Meylan, Burns *et al.* 2004, Cusson-Hermance, Khurana *et al.* 2005). However, TRIFs association with TRAF3 can also stimulate the IRF3 response. TRAF mediated association with TRAF family member-associated NF κ B activator (TANK) protein facilitates the recruitment of TBK1, which phosphorylates IRF3 transcription factor leading to its activation (See 1.1.4.1) (Yamamoto, Sato *et al.* 2003). Furthermore, there is a degree of TRAF-mediated crosstalk between IRF3 and NF κ B pathways with TRAF3 and TRAF6 being able to stimulate both (Fang, Jiang *et al.* 2017).

TLR7 and TLR9 are expressed in plasmacytoid dendritic cells and are the primary sensors for viral ssRNA and unmethylated CpG-DNA respectively. In contrast to TLR3 which mediates downstream signalling via TRIF, TLR7 and TLR9 facilitate signal transduction by interacting with MyD88 adaptor protein at the TIR domain. Activation of these TLRs induces MyD88 interaction with a complex of IL1R associated kinase 4 (IRAK)4, IRAK1 and TRAF6 that ultimately leads to the phosphoactivation of IKK β and then NF κ B (Lin, Lo *et al.* 2010, Kawasaki and Kawai 2014). Interestingly, although it is not thought to induce IRF3 activation, TLR7/9 MyD88 piloted signalling transduction does also lead to TRAF6 mediated IRF7 activation via IRAK1 phosphorylation (Randall and Goodbourn 2008).

1.1.5.2 Cytosolic Pathogen Recognition Receptors

Cells possess a cytosolic PRR repertoire that functions independently of the TLRs but are thought to be essential in the control of both RNA and DNA viruses (Randall and Goodbourn 2008, Goubau, Deddouche *et al.* 2013). These cytosolic PRRs include the RNA helicases retinoic acid inducible gene-I (RIG-I)-like receptor (RLR) family including RIG-I, Melanoma differentiation-associated protein 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2). These detect intracellular ssRNA and dsRNA and activate of both IRF3 (Figure 1-1A) and NFκB (Figure 1-2) pathways, thus inducing expression of type-I IFN and inflammatory cytokines (Yoneyama, Kikuchi *et al.* 2004, Kato, Takeuchi *et al.* 2006, Li, Ranjith-Kumar *et al.* 2009, Rehwinkel, Tan *et al.* 2010). Both RIG-I and MDA5 share a high level of structural homology, containing two N-terminal caspase activating and recruiting domain (CARD) domains and a RNA binding DEAD-box helicase domain. Although both induce IFN response to viral dsRNA, it has been proposed that they are functionally distinct as there appears to be specificity in recognition of RNA dependant on the virus (Kato, Takeuchi *et al.* 2006). RIG-I is stimulated by 5' triphosphorylated RNA (Hornung, Ellegast *et al.* 2006), in contrast MDA5 stimulation depends on the length of the RNA strands, with high molecular weight RNA inducing a response (Pichlmair, Schulz *et al.* 2009). When activated the CARD domains of RIG-I and MDA5 undergo K63 polyubiquitin modification forming oligomers (Jiang, Kinch *et al.* 2012). RIG-I and MDA5 then associate with mitochondrial antiviral-signalling protein (MAVS), another CARD domain containing protein, at the mitochondrial membrane resulting in the formation of MAVS oligomers, a crucial step in IRF3 activation (Hou, Sun *et al.* 2011, Zamorano Cuervo, Osseman *et al.* 2018). These oligomers act as a platform for the

recruitment of IRF3, and TRAF3 which in turn acts to recruit TBK1. This brings IRF3 into close proximity to TBK1 thus facilitating phosphoactivation of the transcription factor (Liu, Cai *et al.* 2015, Fang, Jiang *et al.* 2017). In the context of TLR or RLR signalling TBK1 also associates with scaffold proteins TANK, NFκB activating kinase associated protein 1 (NAP1), and similar to NAP1 TBK1 adaptor (SINTBAD) (Sasai, Shingai *et al.* 2006, Ryzhakov and Randow 2007). Furthermore, stimulation of the RLRs induces cross-pathway activation. RIG-I has reported to induce NFκB via MAVS interaction with TRAF6 that subsequently leads to the activation of the IKK complex. (Yoneyama, Kikuchi *et al.* 2004, Kawai, Takahashi *et al.* 2005, Seth, Sun *et al.* 2005, Xu, Wang *et al.* 2005, Yoshida, Takaesu *et al.* 2008).

The discovery of the cyclic-GMP-AMP synthase (cGAS)-STING-TBK1 pathway has revealed a new sentinel in the host cell repertoire, capable of stimulating IFN in response to viral DNA (Figure 1-1C, and Figure 1-2) (Li, Wu *et al.* 2013, Sun, Wu *et al.* 2013, Zhang, Wu *et al.* 2014, Hertzog and Rehwinkel 2020). cGAS binding of cytosolic DNA is independent of sequence. Binding to DNA forms a 2:2 complex and inducing a conformational change in the PRR, enabling the catalysis of cyclic GMP-AMP (cGAMP) from ATP and GTP (Sun, Wu *et al.* 2013, Zhang, Wu *et al.* 2014). cGAMP functions as a secondary messenger, binding to the ligand binding domain of downstream transmembrane protein dimer stimulator of interferon genes (STING), localised at the endoplasmic reticulum membrane (Ishikawa and Barber 2008, Shang, Zhang *et al.* 2019). This interaction induces a conformational change in STING leading to oligomerisation, and recruitment of TBK1, although

whether this is mediated by TRAFs in a manner like that of MAVS and TRIF remains to be seen (Shang, Zhang *et al.* 2019, Zhang, Shang *et al.* 2019, Zhao, Du *et al.* 2019). The exact mechanism of TBK1 activation by STING remains poorly defined. However in a similar manner to MAVS mediated signalling, phosphorylation of STING enables recruitment of IRF3 to the adaptor protein (Liu, Cai *et al.* 2015). Like other cytosolic PRRs, cGAS-STING has also been implicated in the activation of NF κ B mediated by TRAF6 (Abe and Barber 2014).

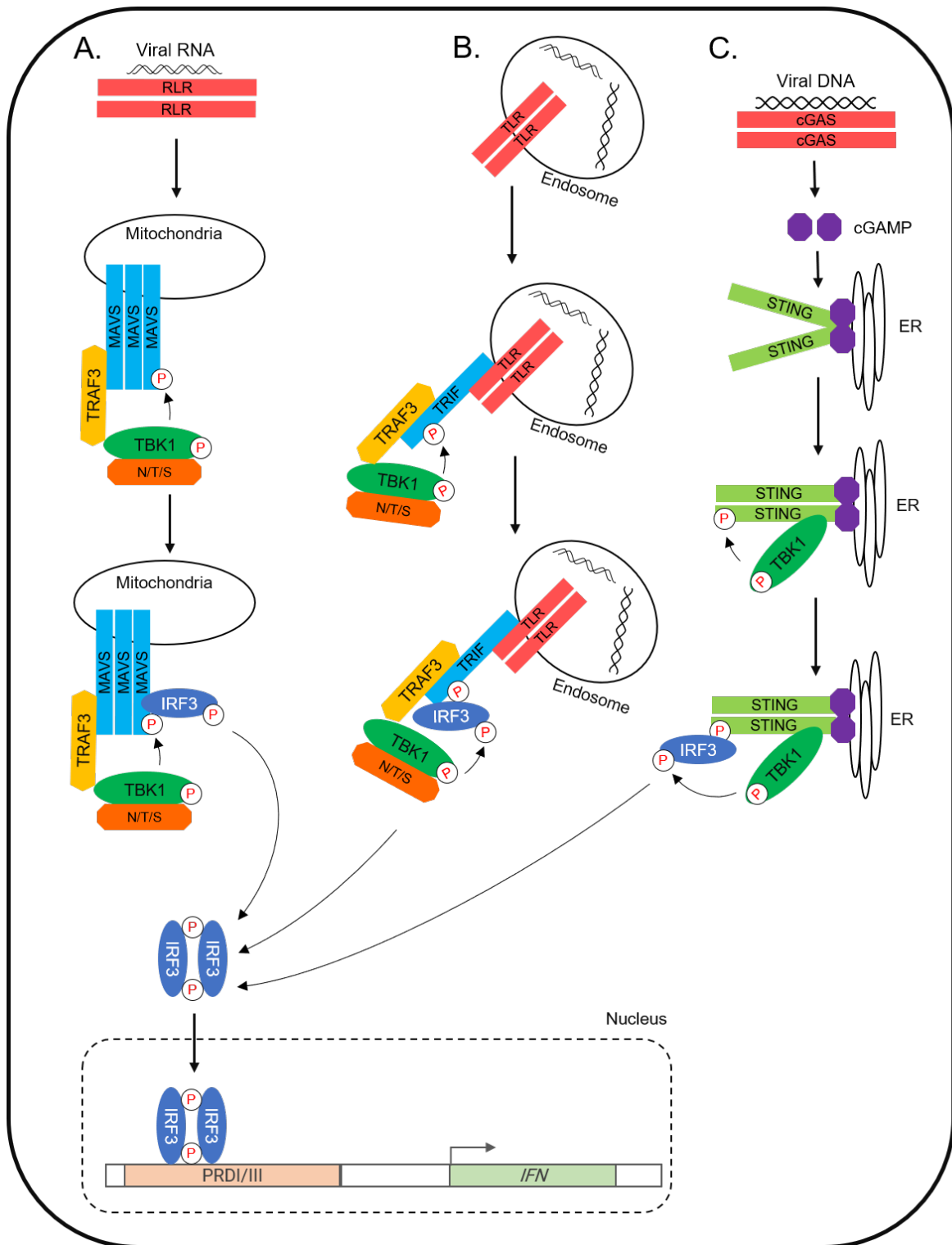


Figure 1-1. Schematic showing an overview of the induction of the IRF3 pathway

A. RLR recognition of viral RNA leads to aggregation of MAVS at the mitochondria recruiting TRAF3 followed by TBK1. TBK1 then phosphorylates MAVS allowing recruitment of IRF3 for phosphorylation by TBK1. **B.** Activation of endosomal TLRs

by viral RNA or DNA recruits TRIF and TRAF3. This in turn recruits TBK1 leading to the phosphorylation of TRIF allowing binding of IRF3 to TRIF and phosphorylation of the transcription factor by TBK1. **C.** Activation of the cGAS pathway by cytosolic DNA induces the production of cGAMP. cGAMP binds to STING at the ER inducing a conformational change which allows binding of TBK1. TBK1 phosphorylates STING enabling the recruitment and binding of IRF3 for phosphorylation by TBK1. **AB.** TBK1 binds to adaptor proteins NAP1 (N), TANK (T), or SINTBAD (S). **ABC.** Activation of IRF3 leads to dimerisation, and nuclear translocation. Schematic adapted from Liu, Cai et al. (2015).

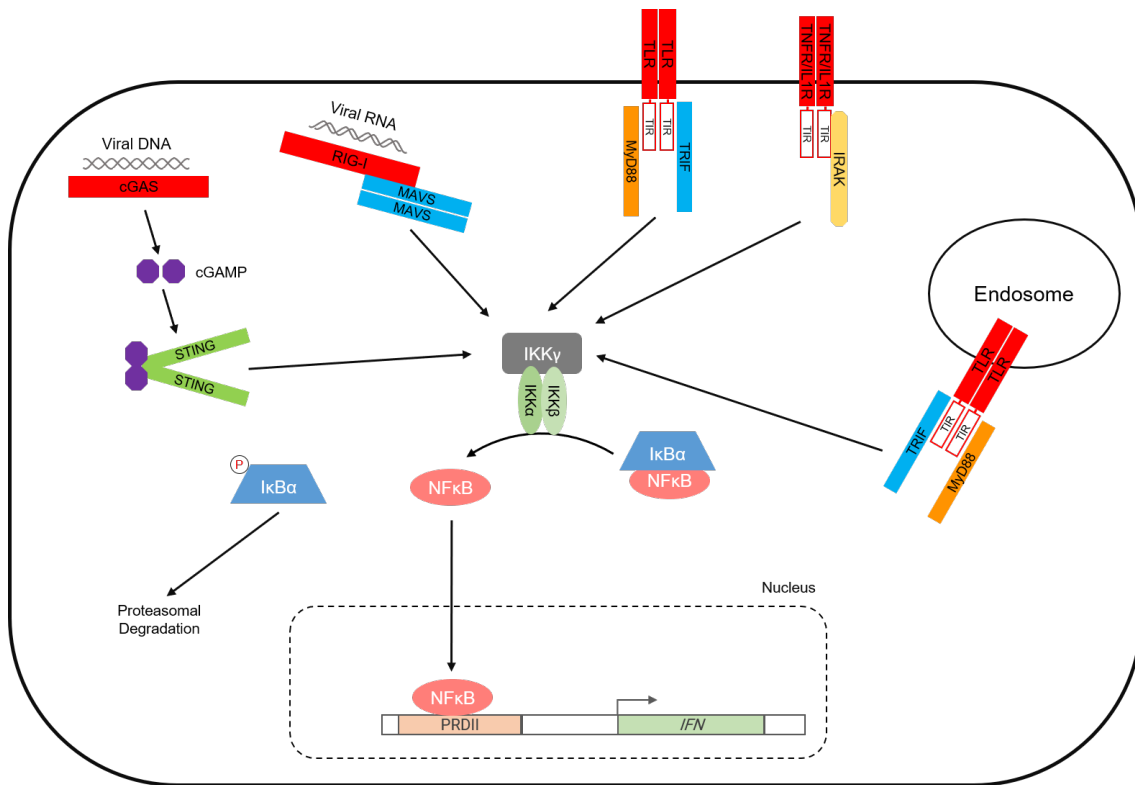


Figure 1-2. Schematic giving overview of NFκB pathway activation Stimulation of various PRRs or cytokine receptors leading to the activation of IKK complex and subsequent IκBα degradation and release of active NFκB.

1.1.6 Interferon Stimulated Genes

Type-I IFN expression activates further signalling cascades and the expression of over 300 genes, known as ISGs (Der, Zhou *et al.* 1998). Binding of secreted IFN to IFNAR1/2 receptor heterodimer facilitates this action. Each subunit of the receptor dimer is associated with different intracellular proteins, IFNAR1 with tyrosine kinase 2 (Tyk2), and IFNAR2 with Janus kinase 1 (JAK)1 but also signal transducers and activators of transcription (STAT)1 and STAT2. Ligand-induced activation of the receptor leads to its dimerisation and Tyk2 mediated phosphorylation of IFNAR1. This brings STAT2 into proximity to the receptor for its phosphorylation. JAK1 then phosphorylates a tyrosine residue of STAT1. The phosphorylated STAT1 and 2 can form a heterodimer via SH domain interaction with phosphorylated tyrosine residues, in the process revealing a novel nuclear localisation signal that allows its nuclear translocation. The STAT1/2 heterodimer binds to IRF9 and forms a heterotrimer known as ISG factor 3 (ISGF3) that binds to the IFN-stimulated response element (ISRE) of ISG promoters to enhance transcription (Randall and Goodbourn 2008). Interestingly cells produce a low basal level of ISGs. A recent model has been proposed indicating that a STAT2-IRF9 heterodimer formed independently of IFN stimulation is retained in the nucleus inducing homeostasis of this low level of basal ISG expression, until IFN stimulation driven formation of ISGF3 acts as molecular switch to enhance ISG expression (Platanitis, Demiroz *et al.* 2019). Type-I IFN driven changes in expression of over 300 genes creates an antiviral environment hostile to the invading pathogen to limit viral replication. The gene profile of this ISG repertoire is highly diverse with a broad range of functions. These include, Ub homologues to disrupt numerous cellular pathways broadly

subduing replication (e.g. ISG15); prevention of vesicle budding (e.g. Myxoma resistance protein (Mx)A); RNase activity limiting viral replication (e.g. 2'-5' oligoadenylate synthetase (2'-5' OAS)); and host cell protein synthesis shut down (e.g. protein kinase R (PKR)) amongst many others (Sadler and Williams 2008). Furthermore, many molecules of the IFN induction pathway are themselves ISGs, this leads to a reinforcement of the response by a positive feedback loop. GTPase proteins MxA/B have been widely implicated in restricting viral replication. MxA protein for instance has been shown to inhibit viral budding by retaining viral nucleoprotein in perinuclear complexes thus limiting viral replication (Kochs, Janzen *et al.* 2002, Netherton, Simpson *et al.* 2009), whereas MxB restricts nuclear import of HIV-1 capsid (Kane, Yadav *et al.* 2013).

Two proteins with a strong impact upon viral replication are the 2'-5' OAS family of proteins and PKR, both are activated by binding dsRNA produced during virus infection and induce RNA degradation, and shutdown of global protein synthesis respectively. Activation of 2'-5' OAS by dsRNA catalyses the production of 2'-5' oligoadenylates (between a dimer and 30mer in length) which bind to ankyrin repeats of endoribonuclease RNase L facilitating its activation and degradation of ssRNA (Dong, Xu *et al.* 1994, Hartmann, Norby *et al.* 1998, Sadler and Williams 2008). Recognition of dsRNA by PKR monomers leads to a conformational shift towards active homodimer configuration inducing PKR mediated phosphorylation of eukaryotic initiation translation factor (eIF) 2 subunit α (eIF2 α) at serine 52 (Dey, Cao *et al.* 2005, Sadler and Williams 2008, Dauber and Wolff 2009, Adomavicius, Guaita *et al.* 2019). eIF2 α is a subunit of the eIF2

guanine nucleotide exchange factor complex which functions to shuttle Met-tRNA to the ribosome, a key component in translation initiation. This is a highly regulated process where phosphorylation of the eIF2 α subunit disrupts the functionality of the complex resulting in inhibition of global protein translation, thus this in turn severely limits translation of viral RNA (Merrick and Pavitt 2018). Moreover, IFN stimulates the expression of a number of genes that may not act to directly limit viral replication, but instead further drive the creation of an antiviral environment at the area of infection. For example, the induction of chemokine and cytokine expression leads to recruitment of immune cells and activation of the adaptive immune response. C-X-C motif chemokine ligand 10 (CXCL10) chemokine secreted by cells binds to the C-X-C motif receptor 3 (CXCR3) receptor in order to drive activation and proliferation of lymphocytes, and direct chemotaxis towards the site of infection (Liu, Guo *et al.* 2011). CXCL10 has been reported to play a significant role in a range of viral infections including limiting coxsackie virus replication, and is highly elevated in influenza, rhinovirus and SARS-CoV-1 infections (Chen and Subbarao 2007, Yuan, Liu *et al.* 2009, Dissanayake, Schäuble *et al.* 2020).

1.1.7 Viral Antagonism of the Type-I IFN Response

The innate immune system plays a key front-line role in eliminating viral pathogens from the host. The selective pressure each one applies to the other drives the evolution of mechanisms of control in the case of the host, but also viral escape mechanisms. Almost every step within the innate signalling pathways have been targeted by viruses with the intent of limiting the host cells' ability to mount an effective antiviral response. It is therefore unsurprising that

viruses have evolved an extremely diverse set of molecular mechanisms to achieve this. As described by Randall and Goodbourn (2008), the mechanisms of action can be broadly divided into 5 key areas: 1 – subversion of IFN induction cascades; 2 – inhibition of IFN signalling; 3 – blocking antiviral ISG products; 4 – interference with global expression patterns; 5 – resistance to the action of IFN. Here I will focus on strategies 1-3.

1.1.7.1 1 - Subversion of IFN Induction Cascades

Viral disruption of IFN induction has been shown to occur at almost every stage in IFN signal transduction pathways including at the level of the IRF3 and NFκB transcription factors, and below. For example repurposing cellular membrane architecture to generate viral factories may compartmentalise PAMPs and thus hide them from host cell defence mechanisms (Novoa, Calderita *et al.* 2005). Respiratory syncytial virus NS2 proteins directly interact with RIG-I via the CARD domain targeting it for proteasomal degradation thus limiting the early induction of IFN. Interestingly NS2 has also been demonstrated to limit TLR3 dependant IFN production at the level of TRIF or below (Ling, Tran *et al.* 2009). Foot and mouth disease virus papain-like proteinase, Leader protease (LPro), targets MDA5 for cleavage abrogating the response (Pulido, Martínez-Salas *et al.* 2020). Moreover, the deubiquitinase activity of LPro shorter variant LbPro has been implicated in the negative regulation of the IFN response by limiting ubiquitination required for activation of a number of key components including RIG-I, TBK1, TRAF3/6 (Wang, Fang *et al.* 2011). Vaccinia virus attenuation of the IRF3 and IRF7 driven IFN response via C6 viral protein is thought to occur at the

TBK1 and IKK ϵ level. The C6 protein co-immunoprecipitates kinase scaffold molecules NAP1, TANK and SINTBAD and thus may block the kinase mediated activation of the transcription factors (Unterholzner, Sumner *et al.* 2011). The cytomegalovirus M45 protein inhibits the NF κ B pathway at a similar level by targeting the master regulator subunit IKK γ of the IKK complex for redirection to autophagosomes leading to its lysosomal degradation. This can prevent the release of the I κ B α negative regulator from NF κ B (Fliss, Jowers *et al.* 2012). Similarly, poxvirus molluscum contagiosum MC160 protein has also been shown to inhibit NF κ B activation via the IKK complex (Nichols and Shisler 2006, Nichols and Shisler 2009). IRF3 and NF κ B transcription factors are also targeted by viral proteins. Classical Swine Fever virus protein NPro targets IRF3 for degradation (Bauhofer, Summerfield *et al.* 2007, Seago, Hilton *et al.* 2007). Whereas Sicilian sandfly virus NSs interacts with IRF3 preventing its binding to the IFN promoter (Wuerth, Habjan *et al.* 2018). Inhibition of the NF κ B transcription factor has similarly been well described. Herpes Simplex virus-1 serine/threonine kinase Us3 hyperphosphorylates p65 preventing its nuclear translocation, and has also been shown to limit upstream TRAF6 polyubiquitination activity (Sen, Liu *et al.* 2013, Wang, Ni *et al.* 2014). Numerous other viral proteins have been shown to antagonise the NF κ B pathway at all levels (Randall and Goodbourn 2008, Deng, Zeng *et al.* 2018). Furthermore, inhibition of the cellular transcription machinery at a level beneath the transcription factors themselves has been shown. Targeting of cofactors CBP/p300 by Bovine HSV ICP0, and Porcine epidemic diarrhoea virus Nsp1 has been demonstrated to interrupt the enhanceosome formation (Zhang, Jiang *et al.* 2006, Zhang, Shi *et al.* 2016). Whereas Thogoto virus ML protein re-localises PIC component TFIIB to create an environment

depleted of the general transcription factor thus limiting the *de novo* recruitment of RNAPII to the IFN promoter (Haas, Meiler *et al.* 2018).

1.1.7.2 2 - Subversion of IFN Signalling Pathway

Targeting of the signalling pathway of IFN itself is another obvious step in the viral arsenal to limit ISG expression. Targeting of STAT proteins has a substantial effect in preventing IFN driven gene transcription. RSV NS1 and NS2 mediate inhibition of STAT2 signal transduction to modulate the IFN response (Lo, Brazas *et al.* 2005, Ramaswamy, Shi *et al.* 2006). This is in addition to the impact on RIG-I by NS2 as previously mentioned, again highlighting the pleiotropic effect of many viral proteins. NS5 of dengue virus, which is known to have RNA-dependant RNA-polymerase properties binds STAT2 inhibiting its phosphoactivation and it has also been implicated in its degradation (Ashour, Laurent-Rolle *et al.* 2009, Mazzon, Jones *et al.* 2009). Similarly, Rabies virus P protein interacts with the DBD coiled-coil region of STAT1, and also STAT2. Interestingly P protein did not prevent phosphoactivation of STAT1/2 but prevented their nuclear translocation. (Vidy, Chelbi-Alix *et al.* 2005, Brzózka, Finke *et al.* 2006).

1.1.7.3 3 - Blockage of Antiviral ISG Products

In addition to attempting to block the IFN response prior to ISG expression, many viruses have evolved mechanisms to directly interfere with or evade the action of ISGs expressed. Protein synthesis shutdown inducer PKR for example is an attractive target for subversion, and is constrained by orthomyxoviruses, Rift Valley fever virus, Ebola virus, amongst others (Dauber and Wolff 2009). Influenza A recruits a cellular inhibitor of PKR P58IPK, whereas HCMV pIRS1 and pTRS1

bind dsRNA, both ultimately blocking the enzyme (Goodman, Smith *et al.* 2007, Bierle, Semmens *et al.* 2013). The ISG apolipoprotein B mRNA-editing enzyme, catalytic polypeptides (APOBEC) are a well characterised family of enzymes involved in restriction of retroviruses. HIV-1 however has evolved a mechanism to subdue their mRNA editing activity. Vif forms complexes with E3 CRL suppressor of cytokines (SOCS)-Box machinery to target the APOBEC3 substrate for ubiquitination and subsequent degradation (Kobayashi, Takaori-Kondo *et al.* 2005, Shirakawa, Takaori-Kondo *et al.* 2006). In fact, utilisation of this mechanism of action is common in virulence proteins of large DNA viruses and is discussed in section 1.1.7.4.

Overall, it is patently clear that viruses have evolved an incredible arsenal of often pleiotropic and diverse mechanisms in an attempt to subvert the IFN pathways at all stages for their own advantage. Therefore, investigation into these mechanisms is key in the understanding of how exactly viruses impact the host.

1.1.7.4 Utilization of Cullin-RING-Ligase Machinery by IFN Pathway Modulators

Interestingly large DNA virus' virulence proteins often deploy ankyrin repeat motifs alongside E3 ligase cullin-RING-Ligase (CRL) machinery recruitment motifs to target substrate proteins for degradation (Sonnberg, Seet *et al.* 2008, Sonnberg, Fleming *et al.* 2009). The CRL is an E3 Ub ligase complex formed of a substrate binding protein, a scaffold cullin (Cul), a variable adaptor protein, a RING E3 ligase protein and an E2 ligase (Figure 1-3) (Petroski and Deshaies 2005, Lydeard, Schulman *et al.* 2013). The substrate binding protein binds to the target

substrate bringing it in proximity for ubiquitination by the CRL and subsequent proteasomal degradation. 5 subclasses of CRL variants are thought to exist, each with a differing profile of components that form the molecular architecture in an almost modular fashion (Petroski and Deshaies 2005, Lydeard, Schulman *et al.* 2013). The specific class recruited by the substrate binding protein is dictated by a particular amino acid motif. This determines which adaptor proteins and which of the seven cullins (Cul1, 2, 3, 4a, 4b, 5, 7) are recruited, this in turn then determines the RING E3 ligase (RBX1 or RBX2) recruited. For example, the presence of what is known as an F-Box motif will recruit SKP1 adaptor protein, Cul1, RING protein RBX1, and an E2 ligase (Figure 1-3A). The SOCS-Box motif will recruit ElonginB (EloB) ElonginC (EloC) heterodimer adaptor, Cul5 scaffold, RBX2 RING and E2 ligase (Figure 1-3B) (Petroski and Deshaies 2005, Lydeard, Schulman *et al.* 2013). Whereas the highly similar Von Hippel-Lindau (VHL)-Box motif recruits the same proteins with the exception that Cul2 is recruited rather than Cul5 (Figure 1-3C) (Kamura, Maenaka *et al.* 2004, Lydeard, Schulman *et al.* 2013).

Substrate binding proteins, containing the motifs to recruit CRL machinery, are widespread throughout the eukaryotic proteasome and have diverse roles. Furthermore binding to the substrate is facilitated by various mechanisms including ankyrin (ANK) repeats, WD40 repeats, leucine rich repeats and other motifs (Petroski and Deshaies 2005). At least 68 F-Box proteins have been described in humans, each with unique interaction domains and therefore varying substrates (Jin, Cardozo *et al.* 2004). The VHL-Box class of CRL derives their

name from the Von Hippel-Lindau tumour suppressor protein, which competes with components of the transcription related protein Elongin (SIII) for EloB and EloC impeding transcriptional activity (Duan, Pause *et al.* 1995, Aso, Haque *et al.* 1996). Interestingly the SOCS-Box motif was originally found in the protein of its namesake, this family of proteins act by limiting the JAK-STAT signalling pathway by recruiting CRL machinery to target these components for proteasomal degradation thus regulating cytokine and ISG expression. However, it is important to note that the presence of a SOCS-Box motif alone does not necessarily indicate a protein's capacity to suppress cytokine activity, as it is the substrate binding domain that dictates target specificity (Krebs and Hilton 2001).

The CRL machinery has been hijacked by multiple viruses to redirect the Ub ligase activity (Mahon, Krogan *et al.* 2014). The best described examples are the poxvirus ankyrin repeat F-Box proteins. This class of proteins possess a novel truncated F-Box-like motif at the N-terminus, adjacent to a string of ANK repeats (protein – protein binding motif) termed pox protein repeats of ankyrin at C-terminal (PRANC) motif, which recruits SKP1, Cul1, and RBX1 CRL machinery to redirect Ub ligase activity (Sonnberg, Seet *et al.* 2008, Chang, Hsiao *et al.* 2009, Sonnberg, Fleming *et al.* 2009, Buttigieg, Laidlaw *et al.* 2013, Noel, Kang *et al.* 2014). Numerous variations between poxvirus PRANC motifs have been observed. One of the shortest which retains functional activity is the 13 amino acid residue of Cowpox CP77 (Chang, Hsiao *et al.* 2009). It is of note that most poxvirus ANK repeat proteins contain a F-Box (Mercer, Fleming *et al.* 2005, Sonnberg, Seet *et al.* 2008). In fact, Sonnberg, Seet *et al.* (2008) reported that 67%

of Avipoxvirus, and up to 100% of Suipoxvirus and Yatapoxvirus ANK proteins contained a PRANC motif. PRANC proteins have been repeatedly demonstrated for the ability to bind to the Ub ligase complex, despite this, limited work has demonstrated their capacity of protein-protein interactions driving degradation of a specific substrate (Herbert, Squire *et al.* 2015). This may be explained if rapid degradation of interactors prevents their observation. However, cowpox 006 protein was shown to control NFκB response by binding and stabilizing the transcription factor precursor p105 preventing its degradation. Interestingly the deletion of the 006 gene was unable to inhibit the NFκB response relative to its parent virus (Mohamed, Rahman *et al.* 2009, Mohamed, Rahman *et al.* 2009). Liu, Olano *et al.* (2019) reported the ability of vaccinia early protein C9 to bind and degrade various IFN-induced proteins with tetratricopeptide repeats (IFITs) via ANK repeats and the recruited SCF complex, thus preventing IFN inhibition of viral replication. Deletion of the C9 F-Box prevented the IFIT degradation. Intriguingly in addition to the F-Box, Odon, Georgana *et al.* (2018) revealed for the first time a novel class of highly conserved poxvirus ANK proteins containing what is known as a BC-Box, a constituent part of the VHL-Box motif that facilitates binding to adaptor proteins EloB EloC heterodimer. The proteins investigated were able to bind Cul2. This suggests that the ability of poxviruses to repurpose the CRL is not just limited to the F-Box SKP1 Cul1, RBX1 CRL machinery but may also be able to hijack the VHL CRL machinery.

Due to the presence of poxvirus proteins that hijack and redeploy cellular CRL machinery for their own benefit, often with the intent of subduing the IFN

response, it would be reasonable to suggest that the same may be true in other large DNA virus families in the Phylum “Nucleocytoviricota” (ICTV Master Species List 2019.v1).

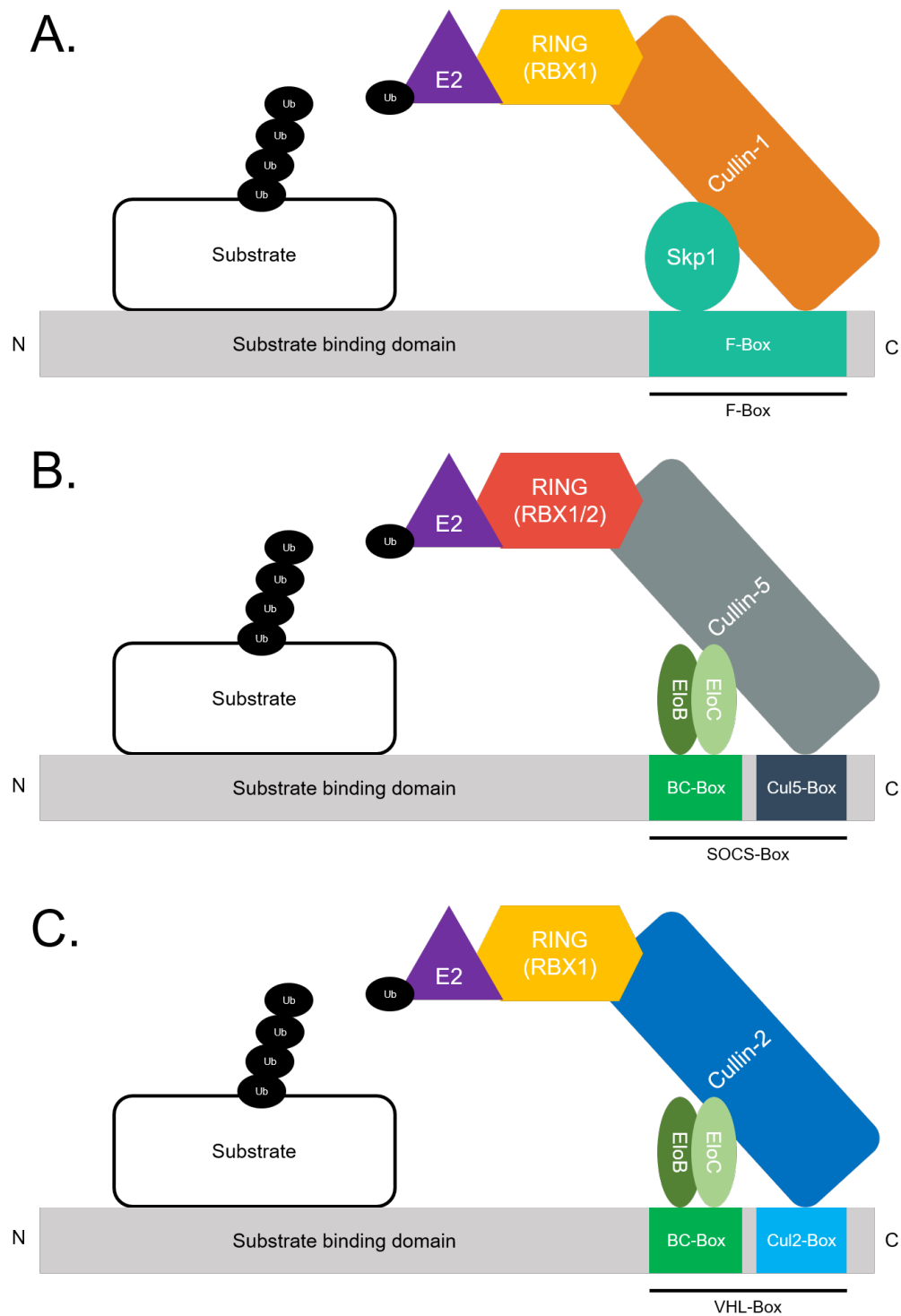


*Figure 1-3. Structure of a substrate binding protein bound to the CRL machinery and substrate **ABC**. Highlights the difference between proteins containing the (A.) F-Box, (B.) SOCS-Box, and (C.) VHL-Box motifs and the division of the VHL/SOCS-Box motif into the two key components, the BC-Box, and the Cul-Box. Adapted from Petroski and Deshaies (2005).*

1.2 African Swine Fever

ASF is a haemorrhagic viral disease of animals of the *Sus* genus, with an extreme lethality of up to 100% in domestic swine and wild boar, thus posing potentially devastating socio-economic risk to regions and peoples that rely on pork production. The causative agent of the disease is the ASFV, a large double stranded DNA virus with a genome of approximately ~170-194kb encoding for ~150-170 open reading frames (ORFs) (Chapman, Tcherepanov *et al.* 2008, Alonso, Borca *et al.* 2018, Cackett, Matelska *et al.* 2020). The virus is the sole member of the *Asfviridae* family. Together with families of other large double-stranded DNA viruses (dsDNA), sharing biological features such as replicating in the cytoplasm of their host cell, including Poxviridae and Mimiviridae, the Asfarviridae have only recently been classified into the Phylum “Nucleocytoviricota” (ICTV Master Species List 2019.v1), and collectively known as Nucleocytoplasmic large DNA viruses (NCLDV) (Walker, Siddell *et al.* 2020). In Eastern and Southern Africa, the virus is present in natural hosts warthog, and soft bodied ticks of the *Ornithodoros* genus where it persists in a sylvatic cycle between the two hosts. Interestingly infection of warthogs produces asymptomatic infection. Furthermore, ASFV is capable of asymptotically infecting other members of the *Sus* genus including the red river hog, and bush pig. However, most importantly infection of wild boar, and domestic swine with highly virulent isolates results in an invariably fatal pathology.

1.2.1 Epidemiology

ASF was first noted in the early 20th century in Eastern Africa and (Penrith, Vosloo *et al.* 2013) remains endemic in sub-Saharan Africa, continuing to pose a

significant risk to rural economies. The virus has since spilt over from Africa into Europe and Asia (Figure 1-4). The first such instance was an introductory event to Portugal in 1957 and again in 1960 where it became endemic to the Iberian Peninsula (Boinas, Hutchings *et al.* 2004, Penrith, Vosloo *et al.* 2013). The presence of the virus was driven by the establishment of ASFV in local tick populations, and free-range farming practices that enabled exposure to the virus (Boinas, Hutchings *et al.* 2004, Penrith, Vosloo *et al.* 2013). The virus remained in pig populations of the region until eradication in the 1990's. Furthermore, ASFV has been present on the Italian island of Sardinia since 1978 and remains persistent in the local population of swine (Jurado, Fernández-Carrión *et al.* 2018). Periodic incursions out of Africa have also been reported, including the Caribbean and Brazil, although infection did not persist in these regions due to aggressive eradication measures (Moura, McManus *et al.* 2010). Outbreaks of ASFV were limited to Africa until 2007 where a major introduction event occurred in the Caucasus nation of Georgia. The virus quickly gained a foothold in the region and progressively spread through Russia and continues to do so, reaching as far West as Belgium in 2018. The transmission of the virus through Europe has been predominantly driven by wild boar populations. 2018 saw the first cases in Asia, with ASFV being first reported in the province of Liaoning of North East China, the world's largest pork producer. The virus rapidly spread across the country's domestic swine farms, and within 6 months from the initial outbreak was found in all provinces. To date containment measures have largely failed, and the Chinese pig herd was reduced by an estimated 50%. This roughly equates to 25% of the global pig population. The virus has since spread beyond Chinese borders into the neighbouring countries of Viet Nam, Cambodia, Laos, but also

Philippines, Democratic People's Republic of Korea, Republic of Korea, and others. Many of these nations are particularly dependant on pork as an economic commodity, but also as a major source of protein. ASFV is therefore considered a major threat to regional food security.

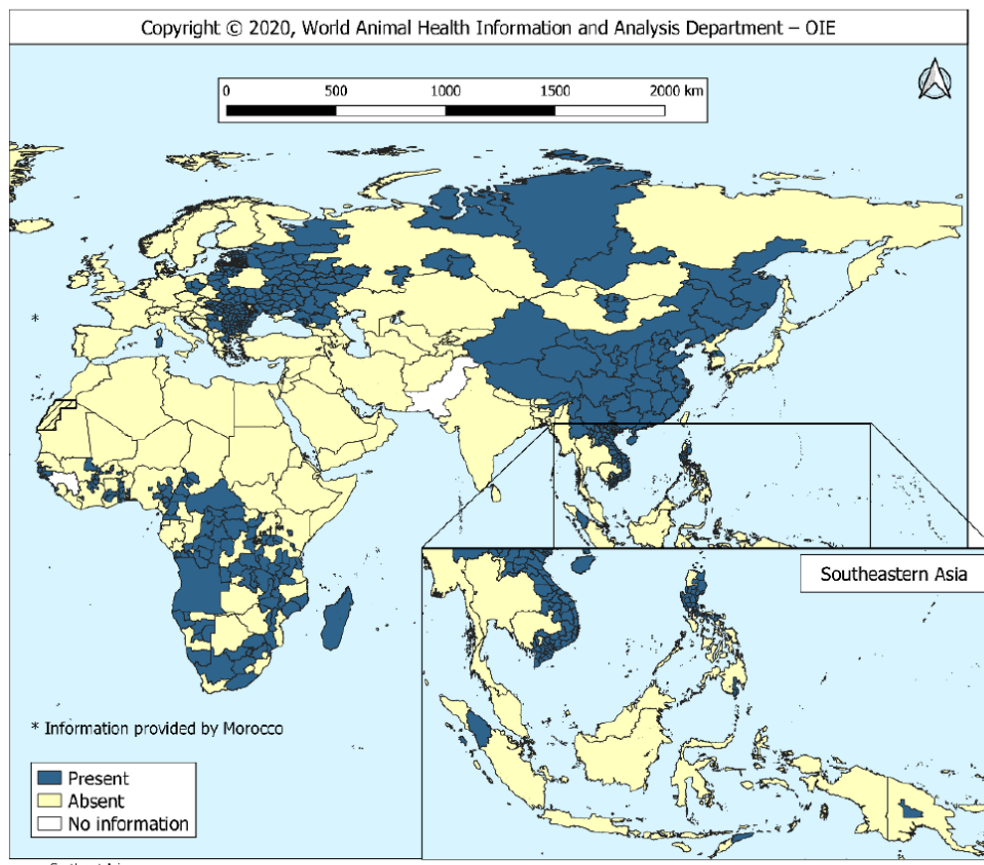


Figure 1-4. Global map of ASFV presence Blue shading indicates countries that have notified OIE of the presence of ASFV from 2016 to June 2020. Adapted from OIE Global Situation report No. 47 (OIE 2020).

1.2.2 Pathology

Infection with highly virulent isolates in domestic swine leads to a characteristic pathology typical of haemorrhagic infection. Peracute rapid clinical course of disease results in high fever above 42°C; lethargy; anorexia and sudden death within 4 days post infection; but limited internal pathology relative to acute infection. Acute infection however is typically more prolonged with clear symptoms similar to that of peracute infection, and death of up to 100% of animals within 7 -14 days post onset, and thus the typical clinical course noted in farms. Animals develop similar external symptoms as in peracute infection, but with addition of cyanosis, most easily noted in the pinnae. Pigs will also display bloody vomiting and diarrhoea, and behavioural changes including huddling together. Post-mortem examination reveals typical internal pathology including but not limited to haemorrhagic splenomegaly, renal petechiae, ascites, and extensive haemorrhagic lymphadenitis. Infection with mildly virulent isolates induces a similar yet more subdued course of disease, although lethality remains up to 70% of infected within 7-20 days post onset (Salguero 2020). Finally, infection with low virulence isolates has been reported to present with a chronic non-lethal form of the disease with clinical manifestations including multifocal necrosis, joint swelling, respiratory distress, and abortive pregnancy (Salguero 2020).

1.2.3 Virology

1.2.3.1 Viral Structure

ASFV is a large icosahedral virus of approximately 250nm in diameter with a highly complex multilayer architecture (Figure 1-5). The virion itself is formed of five layers, the outer membrane, capsid, inner membrane, core shell and nucleoid (Salas and Andrés 2013, Wang, Zhao *et al.* 2019, Andrés, Charro *et al.* 2020). The outer membrane is acquired as progeny virus buds out of the cellular plasma membrane during egress and contains a CD2-like protein, CD2v, that facilitates haemadsorption to erythrocytes (Rodríguez, Yáñez *et al.* 1993, Salas and Andrés 2013). The capsid is formed of the major capsid protein p72 which adopts a jelly-roll β -barrel structure, and over 50 other capsid proteins including minor capsid proteins p17 and M1249L that are believed to play a key role in capsid organisation (Wang, Zhao *et al.* 2019). Cryo-EM analysis revealed that the capsid consists of 2760 pseudo-hexameric capsomers and 12 pentameric capsomeres, with each individual capsomere containing 360 and 90 copies of p72 respectively, in a hexagonal lattice (triangulation number $T=277$) forming the icosahedral structure (Liu, Ma *et al.* 2019, Liu, Luo *et al.* 2019, Wang, Zhao *et al.* 2019, Andrés, Charro *et al.* 2020). The inner lipid membrane, which is thought to originate from the endoplasmic reticulum, contains the transmembrane viral proteins including p17 (D117L gene) and is believed to facilitate encompassment by the capsid (Suárez, Gutiérrez-Berzal *et al.* 2010, Wang, Zhao *et al.* 2019). The thick 30nm core shell is formed of 180 “propeller-like” capsomers, and 12 “starfish-like” pentons in a $T=19$ icosahedral lattice and encompasses the nucleoid (Wang, Zhao *et al.* 2019). Core formation is dependent on processing

of the pp220 polypeptide, precursor to four major structural proteins. Repression of the CP2475L gene, encoding the polypeptide, results in the formation of coreless virions (Andrés, García-Escudero *et al.* 2002). Finally, the 80nm nucleoid contains the ASFV genome, but also the histone-like DNA binding protein pA104R, and transcriptional machinery (Salas and Andrés 2013).

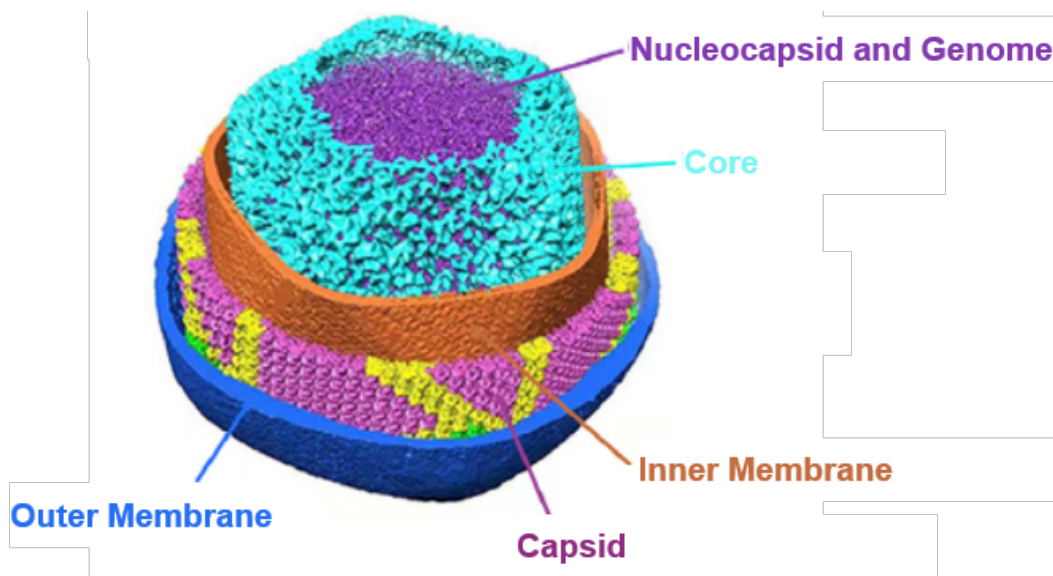


Figure 1-5. Overview of ASFV structure Schematic was adapted from Liu, Luo *et al.* (2019)

1.2.3.2 ASFV Genomics

Within the nucleoid ASFV packages an approximately ~170-194kb linear dsDNA genome, encoding for up to ~150-170 ORFs (Figure 1-7) (Chapman, Tcherepanov *et al.* 2008, de Villiers, Gallardo *et al.* 2010, Cackett, Matelska *et al.* 2020). The function of around 50% of the ORFs remains elusive. The variation in genome size is dependent on the genetic variation of individual isolates which either lack or possess certain genes. This is predominated by variation in the composition of MGF genes, many of which are associated with virulence. These are 5 clusters of multiple copies of paralogues genes at the terminal ends of the genome, that are thought to have arisen by gene duplication. The MGFs will be discussed in detail in 1.2.4. The linear genome encodes for genes in both directions without strand bias (Dixon, Chapman *et al.* 2013). ASFV unique gene nomenclature is based on the first genome sequenced, the VERO cell adapted BA71V isolate. On the basis of *EcoRI* restriction enzyme fragmentation each gene was assigned an initial letter, a number corresponding to amino acid length, and finally L or R indicating orientation. However, this system became unwieldy when applied to what became known as the MGFs due to their wide range of variation between isolates. Chapman, Tcherepanov *et al.* (2008) expanded upon this initial nomenclature and cemented the MGFs into five clusters based on homology, with numbers assigned for amino acid length MGF100, MGF110, MGF300, MGF360, and MGF505. Individual genes of each cluster are further assigned an identifying number, and L or R dictating orientation, e.g. MGF360-12L. Each gene is under the control of a short A T rich promoter that drives a cascading temporal expression; resulting in gene expression being defined as either early, intermediate, or late (Rodríguez, Salas *et al.* 1996, Cackett, Matelska *et al.* 2020).

20% of the genome is dedicated to transcriptional machinery, facilitating independence from the host cellular machinery (Rodríguez and Salas 2013). The viral genome encodes for a range of proteins that form a viral DNA dependant RNA polymerase that resembles the eukaryotic RNAPII complex. This is achieved through the expression of a number of proteins homologous to cellular components; including NP1450L and EP1242L that correspond to RPB1 and RPB2 respectively; and additionally, transcription factor homologues such TFIIB (C315R) and TFIIS (I243L) (Rodríguez and Salas 2013, Cackett, Sýkora *et al.* 2020).

1.2.3.3 ASFV Replication Cycle

ASFV demonstrates a highly specific cell tropism only infecting porcine macrophages of an intermediate to late stage of differentiation. The viral entry receptor remains a mystery, but the virus cell specificity would imply the receptor is macrophage specific (Popescu, Gaudreault *et al.* 2017). Entry occurs in a low pH dependant manner (Galindo, Cuesta-Geijo *et al.* 2015). Moreover, varying mechanisms of entry have been described, including cholesterol and dynamin dependant clathrin-mediated endocytosis; actin dependant endocytosis hinting to phagocytosis; and macropinocytosis (Figure 1-6 – Step 1)(Sánchez, Quintas *et al.* 2012, Galindo, Cuesta-Geijo *et al.* 2015). Following entry into early endosomes the virus progresses through the endocytic pathway (Figure 1-6 – Step 2) (Cuesta-Geijo, Galindo *et al.* 2012). After 30-60 minutes the virus reaches mature endosomal compartments where low pH instigates capsid disassembly (Figure 1-6 – Step 3), leading to internal viral membrane fusion with the

endosomal membrane, subsequently releasing the viral core particle containing the genome and transcription machinery to the cytoplasm (Figure 1-6 – Step 4) (Cuesta-Geijo, Galindo *et al.* 2012). Interestingly Rab proteins and phosphoinositides which ensure proper functionality and integrity of the endocytic pathway are required in early infection (Cuesta-Geijo, Galindo *et al.* 2012).

Once virion cores enter the cytosol, early gene transcription begins in the cytoplasm using enzymes packaged within the virus core. Early virus transcripts code for proteins required for DNA replication as well as many proteins involved in evading host defences. Replication of the ASFV genome is initiated at later times post-infection (~ 6 hours) in viral factories formed near microtubule organising centres (MTOC) in the perinuclear region (Figure 1-6 – Step 5). The initiation of DNA replication results in a shift of the pattern of gene transcription. Late genes code for proteins including those involved in virus assembly or packaged into virions to initiate the next round of infection (Dixon, Chapman *et al.* 2013). Although replication is almost exclusively performed in the cytoplasm, in situ hybridization evidence suggests an early nuclear stage that peaks at 6 hours post infection but is absent by 12 hours (García-Beato, Salas *et al.* 1992, Rojo, García-Beato *et al.* 1999). Interestingly Ballester, Rodríguez-Cariño *et al.* (2011) revealed that early infection leads to major shift in the nuclear architecture. They described infection with ASFV leading to lamin A/C dephosphorylation and reorganisation by 4 hours post infection; nuclear envelope markers being later found in cytoplasmic viral factories indicating viral

induced redistribution; and disruption and redistribution of essential components of cellular transcription such as RNA Pol II. However, despite suggesting a role for the host cell nucleus in replication, the precise role that it plays in the replication cycle of ASFV remains elusive. Rojo, García-Beato *et al.* (1999) suggested that nuclear *de novo* transcription initiated the production of small DNA fragments, which later form the basis for the cytoplasmic generation of linear dimeric concatemers with “head-to-head” ends. The authors proposed that the cytoplasmic replication of the genome occurs in a similar manner to poxvirus *Vaccinia virus* where head-to-head concatemers are formed prior to genome maturation. In this “rolling hairpin strand displacement replication” model, a nick at a 3’ hairpin generates a 3’ OH end to act as a primer for DNA polymerase. Synthesis of nascent DNA results in strands that are self-complementary and thus fold back in on themselves forming concatemers. The concatemers are finally resolved to produce mature genomes (Moss 2013). Encapsidation mechanism of the ASFV genome is unclear with two models proposed. The first suggests the formation of nucleoprotein complexes in viral factories that are encapsidated into empty virions. The second however suggests the DNA is first encapsidated and then repackaged resulting in mature virus (Figure 1-6 – Step 6) (Salas and Andrés 2013).

Once the mature virion is complete, ASFV is recognised by kinesin utilising microtubules to transport from the perinuclear viral factories towards the plasma membrane (Jouvenet, Monaghan *et al.* 2004). The virus exits the cells by budding from the plasma membrane, acquiring its additional membrane in the process

(Figure 1-6 – Step 7) (Andrés, García-Escudero *et al.* 2001, Salas and Andrés 2013).

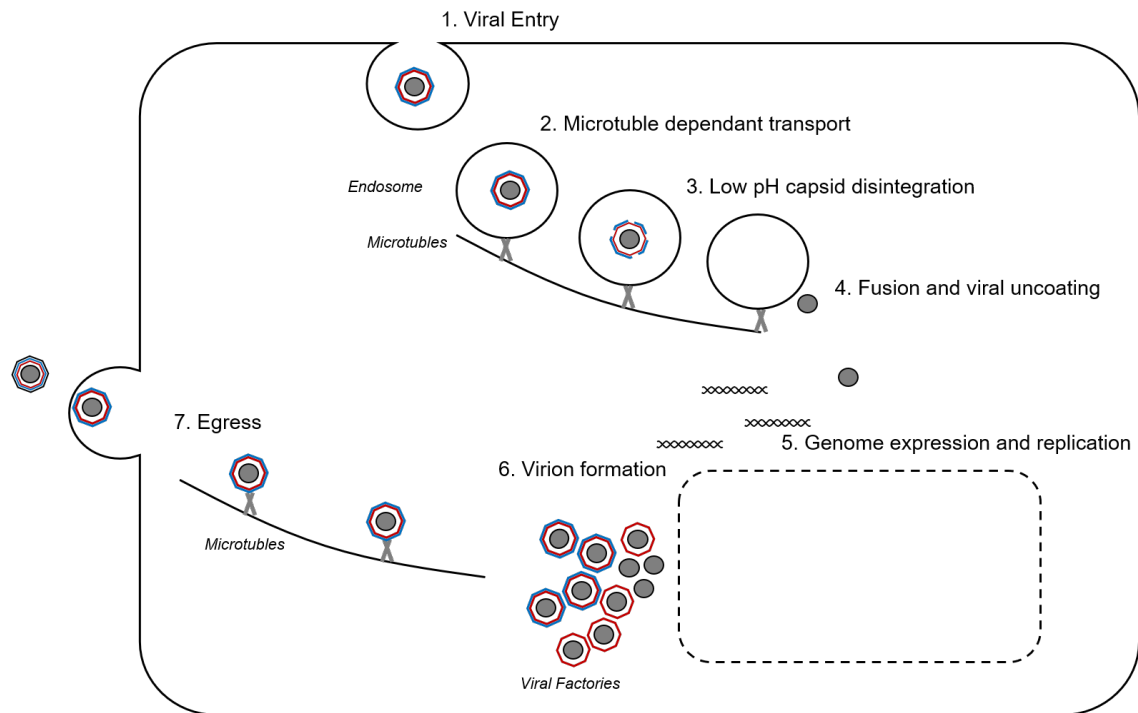


Figure 1-6. Overview of the ASFV replication cycle Diagram was adapted from Galindo and Alonso (2017).

1.2.4 Multigene Family Genes

The MGFs are clusters of multiple paralogous genes (different gene copies found on the same genome) found at the terminal ends of the ASFV genome (Figure 1-7). Approximately 30% of the genome is dedicated to the MGFs, occupying approximately 40kbp and 20kbp of the left and right ends, respectively. The genes can be divided into 5 gene clusters based upon sequence identity and amino acid length, known as MGF100, MGF110, MGF300, MGF360 and MGF505, named after the average number of codons. Many genes of the MGFs appear to play a role in virulence and host immune response modulation, however the specific mechanisms of which remain elusive (Zsak, Lu *et al.* 2001, Neilan, Zsak *et al.* 2002, Afonso, Piccone *et al.* 2004, Burrage, Lu *et al.* 2004, Chapman, Tcherepanov *et al.* 2008, O'Donnell, Holinka *et al.* 2015, Golding, Goatley *et al.* 2016, Reis, Abrams *et al.* 2016, Chen, Zhao *et al.* 2020). Clusters can contain between 2-19 individual paralogs, however, the number of individual genes per cluster differs per isolate, and is the primary explanation of the variation in sequence lengths observed (Chapman, Tcherepanov *et al.* 2008). MGF100 has 3 known paralogs, MGF110 13, MGF300 3, MGF360 19, and MGF505 10. As mentioned above the number of paralogous genes within a cluster is highly variable. This is most evident in MGF360 cluster where a total of 19 genes are observed across different isolates. Low virulent isolate OURT88/3 contains only 9 MGF360 genes, whereas Ken60 contains 17. MGF505 cluster however appears less variable between isolates with 10 paralogs recognised; most isolates contain all 10, but notably attenuated OURT88/3 and NHP/68 are lacking MGF505-1R, 2R and have a partially truncated MGF505-3R variant. Interestingly, a number of isolates including Ken05, Ken1950, Ken06_Bus all have a MGF505-

6R and 7R fusion protein (Imbery 2017). MGFs of the same cluster are typically read in the same direction, and thus are thought to have arisen by a gene duplication method. This may not be too dissimilar from the accordion gene expansion mechanism observed in poxviruses. Due to the high fidelity of replication seen in large DNA viruses it has been proposed that this mechanism is utilised to rapidly overcome selective pressure. It is thought that when under selective pressure from host cell antiviral responses, these poxvirus genes duplicate (the accordion is expanding) to introduce gene variation allowing adaptation; eventually the accordion contracts leaving genes with a selective advantage over the host antiviral response remaining (Elde, Child *et al.* 2012). The OURT88/3 isolate for example has an additional gene belonging to MGF505 cluster; MGF505-8R, that is not observed in other isolates. MGF505-6R and -8R show a high similarity to the adjacent MGF505-7R, thus it has been proposed that they may be the consequence of a recent duplication event (Chapman, Tcherepanov *et al.* 2008). Sequence comparison shows that a potential consequence of gene duplication is that the variation between paralogous genes of a single cluster can be large. For example, MGF360-1L and 18R share only 12% identity; however, functional motifs remain conserved. Furthermore orthologs (a single gene found in different isolates) between isolates show a higher similarity, for example MGF360-8R shares at minimum 86% identity (Dixon, Chapman *et al.* 2013). Curiously, paralogs of the MGF360 cluster found at the 3' end of the genome show a lower sequence divergence than expected, suggesting a recent duplication event originating in gene transposition. In fact an inversion of a large 7kbp region from the 5' end to the 3' end was found in a attenuated isolate from Estonia (Genotype II, Georgia 07-like) resulting in duplication of 10 MGF genes,

suggesting that genome architecture reorganisation is possible (Zani, Forth *et al.* 2018).

Despite the MGFs comprising roughly 30% of the ASFV genome, their function remains somewhat of a mystery. Very little evidence exists explaining the functional role of MGF100, and MGF300 clusters. Confocal microscopy shows that MGF110-4L contains functional endoplasmic reticulum (ER) retention (KDEL) motifs and localises to pre-Golgi compartments, and has been proposed to play a role in viral morphogenesis at the ER (Netherton, Rouiller *et al.* 2004). Most work into the functionality of the MGFs has focused upon genes of the MGF360 and MGF505 clusters. Evidence strongly hints towards many of these proteins in subversion of the host immune response, and host determination. A major clue into the MGF360 and MGF505 functionality was revealed with sequence analysis of the naturally attenuated OURT88/3 isolate; the isolate is lacking a 10kbp region from the left terminal end encompassing MGF360-10L, 11L, 12L, 13L, 14L, MGF505-1R, 2R, and 3R (Chapman, Tcherepanov *et al.* 2008). This isolate is highly attenuated in swine and is capable of inducing a strong IFN response but also shows a reduced replicative ability in macrophages pre-treated with IFN α , whereas the opposite is true for highly virulent isolates containing the genes (Boinas, Hutchings *et al.* 2004, Golding, Goatley *et al.* 2016). Similarly, Pr4 Δ 35 lacking 6 MGF360 and 3 MGF505 genes induced the upregulation of a range of ISG, including MX, and CXCL10 amongst others. The loss of MGF360 proteins also resulted in growth defects. Virulent isolate Pr4 genetically modified to lack MGF360-12L, 13L and 14L demonstrated up to 1000 fold reduction of

growth in ticks (Burrage 2013). Infection of porcine macrophages with this deletion mutant was shown to upregulate the expression of IFN- α but also a range of ISGs including MX, ISG43 and PACT, whereas parental isolate did not, again suggesting MGF360 and MGF505 proteins were subverting the type-I IFN response (Afonso, Piccone *et al.* 2004, Reis, Abrams *et al.* 2016). Some assessment of individual MGF genes has been performed (Correia, Ventura *et al.* 2013). showed that MGF360-15R inhibited TLR3 and RIG-I driven IFN β expression, but not the JAK/STAT pathway. Furthermore the same group demonstrated that MGF505-7R functioned to inhibit the IRF3, NF κ B and the JAK/STAT pathways. Until very recently research investigating the exact molecular mechanisms used by the MGF360 or MGF505 proteins was non-existent. Work examining the late expressed MGF360-16R by Ramírez-Medina, Vuono *et al.* (2020) using yeast-2-hybrid has shown the viral protein interacts with SERTA Domain 3 (SERTAD3) and syndecan-binding protein (SDCBP). SDCBP has been shown to play a role in endocytic trafficking in HPV infection (Gräßel, Fast *et al.* 2016), however very little is known about SERTAD3 with the exception that it has been implicated to play a role as a cofactor in E2F transcription, but also as an ISG limiting influenza virus replication (Darwish, Cho *et al.* 2007, Sun, Li *et al.* 2020). Mutant virulent virus lacking MGF360-16R did not show a reduction of viral growth in cells or attenuation in pigs (Ramírez-Medina, Vuono *et al.* 2020). Very recent work published during the writing of this thesis has probed the mechanism of action of MGF360-12L, where it has been observed to limit mRNA transcription of a wide variety of ISGs in HeLa cells. The researchers proposed that MGF360-12L inhibits the NF κ B signalling pathway by interacting with and

targeting importin α , effectively limiting nuclear translocation of the transcription factor (Zhuo, Guo *et al.* 2020).

The evidence to date clearly suggests that the MGF360 and MGF505 proteins play a role in the attenuation of ASFV, most likely by inhibition of the host innate immune system and IFN response. However, the specific mechanism of interaction remains uncertain, thus warranting further investigation. Furthermore, the role of the MGFs in attenuation make them attractive targets for the development of deletion mutant LAV candidates. Functionally assessing the MGFs to build a deeper understanding of their molecular function will enable a more rational approach to the consideration of genes to target for deletion.

1.2.5 Other Immunomodulatory ASFV Genes of Note

ASFV codes for a number of additional genes involved in inhibition of the type-I IFN response. I329L gene for example encodes for a highly glycosylated protein that acts as a TLR3 antagonist by targeting TRIF to inhibit IRF3 and NF κ B pathways (de Oliveira, Almeida *et al.* 2011). The A238L protein is a known NF κ B inhibitor and limits the production of COX2 and TNF α by preventing CBP/p300 activation (Granja, Nogal *et al.* 2006, Silk, Bowick *et al.* 2007, Granja, Sánchez *et al.* 2009).

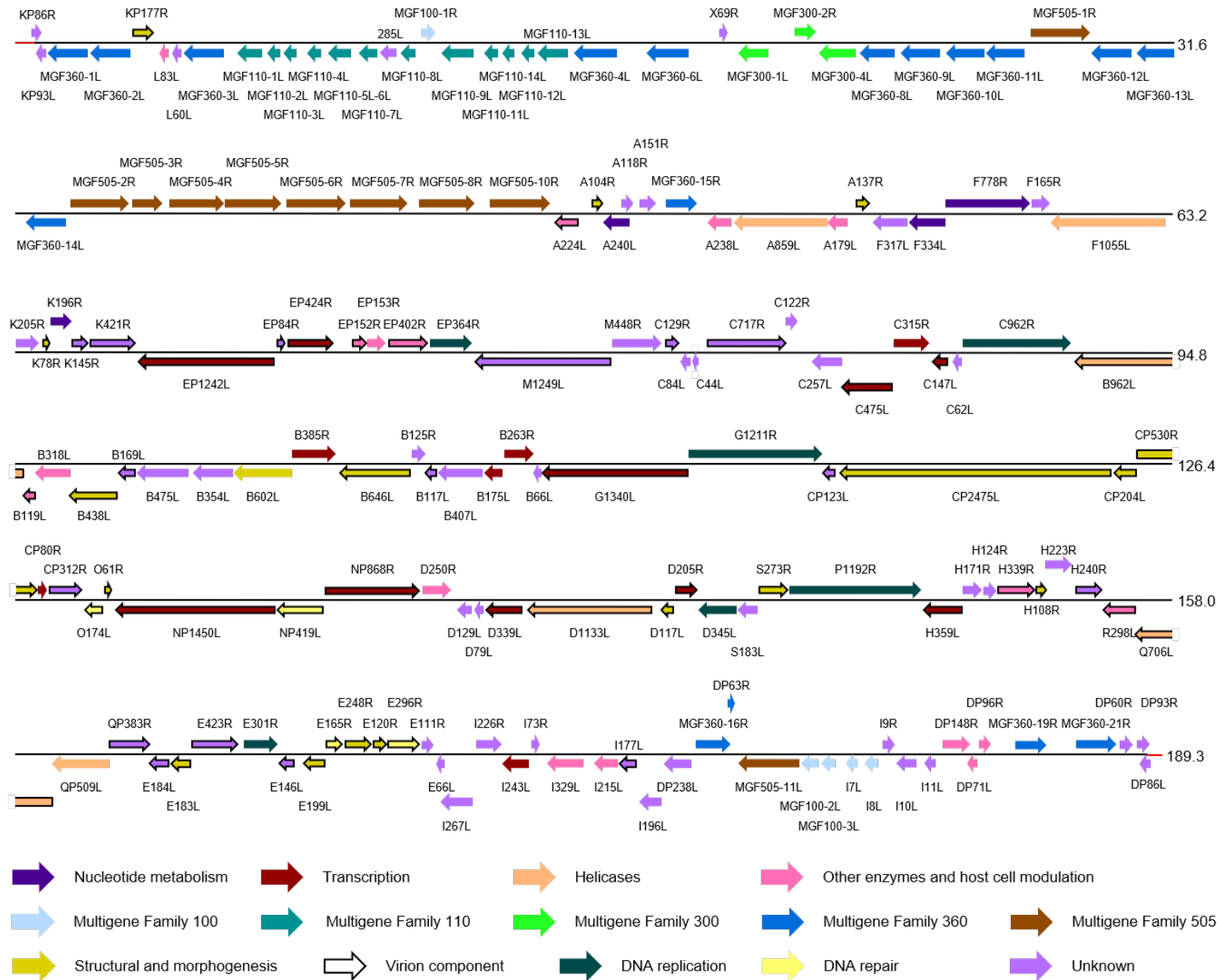


Figure 1-7. Architecture of Georgia07/1 genome

ORFs are shown by coloured arrows, with direction of arrow dictating read direction. Note MGF gene clusters at the terminal ends. Adapted from Dixon (2020).

1.2.6 ASFV Vaccinology

The development of a vaccine to ASFV is a critical advancement required for the effective control of the disease. Currently no ASFV vaccines are licensed, leaving preventative biosecurity measures or extermination the only methods of dealing with outbreaks of the disease. Due to the complexity of the virus and relative lack of global financial interest until fairly recently, progress in vaccine development has been somewhat slow. Large gaps in knowledge of the field remain that gravely hinder progress. Characterisation of the correlates of protection are unclear, and specific mechanisms of virulence remain to be seen (Carlson, O'Donnell *et al.* 2016). It appears that both the induction of a cytotoxic T cell and humoral response are necessary for protection (Onisk, Borca *et al.* 1994, Oura, Denyer *et al.* 2005, Takamatsu, Denyer *et al.* 2013){Denyer, 2006 #543}, although the specific profile of immune response required remains unknown therefore compounding the issue of vaccine evaluation and development.

Furthermore, there are a number of factors to be considered for an 'ideal' ASF vaccine. Safety is obviously paramount, and any vaccine should be proven to pose acceptable levels of risk to not only pigs, but also contact animals (no reversion to virulence), the environment, and finally be fit for human consumption of pork. Moreover, in industrial farming economic factors as well as animal health should be considered with livestock vaccination; febrile illness upon immunization should be as limited as possible due to the negative consequence to animal growth and thus limiting economic yield. However, this would be considered less of a fear for immunisation in resource poor settings due to the

survival of the pigs being the supreme concern for sustenance and smallholder farming. Immunisation should not cause viraemia, as this correlates to pathology, and have no capacity for onwards transmission of the vaccine strain (although this is not relevant for subunit vaccines). A high level of protection is desired (ideally 100% against multiple ASF genotypes), with no subsequent pathology or persistent infection of challenge virus observed and no risk of transmission to contact animals. Finally, from a regulatory affairs standpoint, it is likely that a licensed vaccine needs to conform to DIVA standards to enable differentiation between vaccinated and infected animals.

Despite the obvious and several difficulties in ASF vaccine development, a number of labs have endeavoured to undertake the challenge and some key albeit limited progress has been made, which I will further describe here.

1.2.6.1 Inactivated Vaccines

Inactivated vaccines are a class of vaccines that utilises inert or “killed” pathogenic agents that do not cause pathology with aim of stimulating an immune response. Attempts to use inactivated virus vaccines against ASFV have thus far failed. Initial examinations with inactivated virus were performed in the 1960’s with little protective effect (Stone and Hess 1967). More recently Blome, Gabriel *et al.* (2014) readdressed the issue by applying more modern binary ethyleneimine (BEI) inactivation techniques, combined with “state-of-the-art” adjuvants, Polygen™ or Emulsigen(®)-D. Unfortunately, this was also unsuccessful in conferring protection. In fact immunisation resulted in

accelerated progression of clinical pathology, particularly in animals that mounted the strongest antibody response. This potentially hints towards an antibody dependant enhancement of viral entry consequently leading to more severe pathology (Blome, Gabriel *et al.* 2014). Despite the simplicity of inactivated vaccines making them an attractive target, unfortunately it would appear in the case of ASFV this is unlikely to be a feasible option.

1.2.6.2 Subunit and Virus Vectored Vaccines

More recent attempts at using antigen subunit-based vaccines have shown some promise, albeit with conflicting results (Arias, De la Torre *et al.* 2017). These are a class of vaccines that present one or more specific antigens to the immune system in aim of stimulating a response. This is either by vaccination with isolated antigen that is often recombinantly expressed (antigen vaccine – See 1.2.6.2.1), or via a genetically modified viral vector (e.g. adenovirus vector) (viral vectored vaccine – See 1.2.6.2.2) that is used to deliver genetic material coding for antigen/s to host cells, subsequently inducing the expression of the encoded antigen resulting in an immune response. The use of subunit vaccines would allow a vaccine to conform to differentiation of infected and vaccinated animals (DIVA) a key component for licensing of any prospective product. Furthermore, they offer a theoretically safer alternative than LAVs as they are incapable of potential reversion to virulence. However, as ASFV encodes for up to 170 proteins and the correlates of protection remain unclear the selection of appropriate antigens has proven challenging.

1.2.6.2.1 Antigen Vaccines

Research initially focused upon using recombinantly expressed antigens for vaccination, mostly including viral hemagglutinin protein CD2v, or structural proteins p54 and p30 (Gaudreault and Richt 2019). Vaccination with baculovirus expressed CD2v alongside Freund's adjuvant induced antibody but not T cell response and 100% protection against lethal E75 isolate, although 2/3 of vaccinated pigs displayed viraemia on par with that of control animals. Studies investigating vaccination with p30 and p54 antigens, either alone or in a fusion protein saw an antibody response, and between 50-100% protection with E75 challenge, but also a drop in viraemia compared to control animals (Gómez-Puertas, Rodríguez *et al.* 1998, Barderas, Rodríguez *et al.* 2001). Whereas adding p72 and p22 antigens to the vaccine composition offered a delayed progression of disease but no protection, however in this study challenge was performed with a titre of Pr4 challenge virus 2 orders of magnitude higher (Neilan, Zsak *et al.* 2004). This may provide insight that using antigen vaccination is capable of inducing some level of protection, but that it is insufficient for higher doses of infection.

1.2.6.2.2 Viral Vectored Vaccines

Recent developments in using attenuated and replication deficient viral vectors to induce expression of subunit antigens have shown promise. Many investigations have focused upon structural proteins, CD2v, and others (Gaudreault and Richt 2019). Varied results have been observed with some researchers reporting both robust antibodies and T cell responses but limited

protection (Lokhandwala, Waghela *et al.* 2016, Lopera-Madrid, Osorio *et al.* 2017, Lokhandwala, Petrovan *et al.* 2019, Netherton, Goatley *et al.* 2019). This in essence highlights the primary issue with subunit vaccines currently, as the correlates of protection for ASFV are poorly characterised, the selection of antigen profile is exceptionally difficult (Carlson, O'Donnell *et al.* 2016, Arias, De la Torre *et al.* 2017). Netherton, Goatley *et al.* (2019) however attempted to overcome this by employing a systematic approach to screen 40 ASFV genes for immunogenicity. Pools of 18 viral antigens that were recognised by porcine lymphocytes on infection, were assessed for immunogenicity and protective capacity when expressed by Adenovirus or modified vaccina Ankara (MVA) vectors in pigs. Despite being capable of inducing a cellular and humoral response with homologous and heterologous prime and boost, the antigen pools tested only provided partial protection on challenge (Netherton, Goatley *et al.* 2019). Similarly, Lokhandwala, Petrovan *et al.* (2019) showed pigs were able to induce a strong IgG response to vaccination with adenovirus expressing combinations of up to 9 ASFV genes but were unable to survive challenge with genotype II Georgia 07/1. In contrast however, building upon their previous work the Netherton group showed that the delivery of 8 antigens via Adenovirus and further MVA boost is capable of protecting 100% of pigs from lethal genotype I challenge (Goatley, Reis *et al.* 2020). Interestingly enhanced pathology has been noted with antigen combinations, highlighting the need for their careful selection (Argilaguet, Pérez-Martín *et al.* 2012, Lokhandwala, Petrovan *et al.* 2019, Netherton, Goatley *et al.* 2019). This data shows that viral vectored vaccines pose a feasible and promising development in the race for an ASFV vaccine, however

the correct cocktail of antigens and prime boost regimen remains a critical decision in any potentially successful vaccine.

1.2.6.3 Live Attenuated Vaccines

LAVs are produced by reducing the pathogenicity of an infectious agent (e.g. by modification deleting virulence genes) whilst retaining its viability with the aim of inducing an immune response. These are thought to typically induce a more robust response compared to inactivated vaccines. Most research investigating ASFV vaccines has focused upon LAVs, which are thought to be the vaccine candidates with the best short-term potential. Thus far, a number of LAV candidates have provided strong protection, although many present with low level viraemia or adverse reactions and are therefore not commercially viable (Arias, De la Torre *et al.* 2017).

Vero cell line adapted attenuated isolates were first trailed on the Iberian Peninsula in 1960's, however their use was halted due to an unacceptably high prevalence of chronic pathology (Arias, De la Torre *et al.* 2017). The use of naturally attenuated isolates such as genotype I OURT88/3 has been trialled, although not taken forward due to safety concerns (Sanchez-Cordon, Chapman *et al.* 2017). This isolate provides protection against challenge with lethal Benin97/1 (genotype I), and importantly Uganda 1965 (genotype X) suggesting cross protective capacity (King, Chapman *et al.* 2011). However, pigs inoculated with OURT88/3 have been reported to present with persistent viraemia, chronic pathology, and clinical signs post challenge, despite protection (Sanchez-Cordon,

Chapman *et al.* 2017). Moreover, vaccination with naturally attenuated isolates NH/P68 has shown adverse reactions, including clinical presentation typical of chronic infection such as fever and lesions (Leitão, Cartaxeiro *et al.* 2001). Concerningly, NH/P68 could be isolated up to 99 days post inoculation and was capable of transmission to non-vaccinated naïve pigs (Gallardo, Soler *et al.* 2015).

Genetic modification of virulent ASFV isolates to delete immunomodulatory virulence genes has been a common method of attenuating the virus in aim of LAV development. Removal of the early transcribed but yet uncharacterised DP148R gene attenuated Benin97/1, and provided 100% protection against lethal challenge, however animals showed unacceptable levels of transient fever postimmunisation (Reis, Goatley *et al.* 2017). Deletion of the uncharacterised I177L gene from Georgia07/1 completely attenuated the lethal isolate, and furthermore provided a strong and sterile protective effect against low dose challenge with the parental virus (Borca, Ramirez-Medina *et al.* 2020). Deletion of EP402R encoding viral hemagglutinin protein CD2v thought to promote viral transmission attenuated virulent BA71 and protected pigs against the parental background, E75 isolate of the same genotype (genotype I), and Georgia07/1 (genotype II) providing further evidence that cross protection is possible (Monteagudo, Lacasta *et al.* 2017). In contrast, deletion of EP402R from genotype II Georgia2010 did not attenuate the virus (Borca, O'Donnell *et al.* 2020). Likewise, deletion of 9GL and UK from Georgia07 attenuated the virus and offered homologous protection, whereas the same deletion in the highly similar Chinese

isolate HLJ/18 did not (O'Donnell, Risatti *et al.* 2017, Chen, Zhao *et al.* 2020). Taken together these data suggests that gene deletion is capable of attenuating lethal ASFV and inducing protection. However, there are inconsistencies between which gene deletions are sufficient for attenuation between differing isolates and whether heterologous genotype protection is possible.

The discovery that MGF360 and MGF505 genes are virulence factors and responsible for the attenuation of OURT88/3 (Discussed in 1.2.4) has led to these genes being a strong focus of vaccine development. Deletion of the MGF360-10L, 11L, 12L, 13L, 14L, MGF505-1R, 2R and 3R alongside interruptions in MGF360-9L and MGF505-4R from genotype I Benin97/1 produced a replication competent deletion mutant that was attenuated in pigs. Vaccination and boost with this candidate produced a short transient fever, but no clinical pathology after challenge (Reis, Abrams *et al.* 2016). Furthermore deletion of 6 MGF genes from the lethal Georgia07/1 background attenuated the virus and conferred protection (O'Donnell, Holinka *et al.* 2015), however the additional deletion of the 9GL gene, performed in an attempt to increase safety, did not provide protection (O'Donnell, Holinka *et al.* 2016). Although previous results showing that the deletion of 9GL alone did not attenuate the virus, when combined with the MGFs the authors saw a restricted growth phenotype in macrophages (O'Donnell, Holinka *et al.* 2016). This suggests the additional deletion further “weakened” the isolate thus in essence over attenuating the candidate, preventing the animals from mounting a sufficient immune response. This highlights the need to find the correct balance between attenuation and immunogenicity. Most recently Chen, Zhao *et al.* (2020)

showed that deletion of MGF360-12L, 13L, 14L, MGF505-1R, 2R, and 3R, from HLJ/18, an isolate highly similar to Georgia07/1, did not cause fever post immunisation and protected 100% of pigs challenged, but results raised safety concerns. Further understanding of the functionality of the MGFs in question may provide a deeper insight to more appropriate profile for deletion.

As with all LAVs there are additional safety concerns to consider, particularly the reversion to virulence, but also the potential for vaccine strains to progress into chronic pathology. A single gene deletion mutant may pose a higher risk of reversion to virulence, therefore multiple deletions may provide a safer alternative. However a major concern was raised with the results of Chen, Zhao *et al.* (2020) who reported a reversion too virulence in HLJ/18 lacking MGF360-12L, 13L, 14L, MGF505-1R, 2R, and 3R after 5 passages through pigs. Disappointingly they did not publish any sequence data for the virus isolated after these passages.

There is sufficient evidence to suggest that LAVs offer a potential successfully route for prophylaxis of ASFV. However, a number of questions remain; conforming to the required DIVA standards is obviously more of a challenge compared to other vaccine systems, but can be overcome by deletion of a gene known to produce an antibody response; evidence of heterologous genotype protection, and even homologous genotype protection is inconsistent; and furthermore, large questions remain regarding the safety profiles of the candidates. Currently further investigation is required to fully understand the

impact of gene deletions on attenuation of the virus and efficacy of protection offered.

1.2.7 Aims of the Project

Primary Aims

- * Functionally assess the impact of individual MGF360 and MGF505 proteins on the NFκB and IRF3 pathways
- * Characterise inhibitory mechanism of individual MGF360 and MGF505 proteins

Secondary Aims

- * Use functional knowledge gained to rationally select MGF genes for deletion for LAV candidates

2 MATERIALS & METHODS

2.1 Computational Bioinformatics Analysis

Computational analysis was performed with a variety of online software (Table 2-1). Default settings were used unless stated otherwise in the text.

Software	Application	Notes
BLASTp: Basic Local Alignment Search Tool	Comparison of protein sequences to databases	Comparative searches excluded African swine fever virus (taxid:10497)
EMBI – Clustal Omega	Multiple sequence alignment	
EMBL-EBI PFAM Motif Finder V.33.1	Protein motif finder	
Expasy – ScanProsite	Protein motif scanner	
Expasy - SWISS-MODEL ProMod3	Protein homology-modelling	
HMMER V.3.3 HMMscan	Protein homology searching	Search against PFAM HMM Library
Jpred V.4	A Protein Secondary Structure Prediction Server	

Table 2-1. Software used in computational analysis

2.2 Cell Culture

2.2.1 Cell Lines

HEK293T, HeLa, and A549 cells were supplied by The Pirbright Institute's internal cell bank. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) high glucose GlutaMax™ (Gibco) 10% Foetal calf serum (FCS) (Life Science Production), 100 U.mL penicillin streptomycin (PS) (Gibco) in T175 flasks (GreinerBio-One), at 37°C, 5% CO₂, and 95% humidity. Cell morphology was regularly visually inspected. Cells were passaged at approximately 80% confluency. For passage, cells were washed once in PBS and incubated in 0.05% trypsin- Ethylenediaminetetraacetic acid (EDTA) (Gibco) and re-suspended at appropriate density dependant on assay ($5 \times 10^4 - 6 \times 10^5$).

2.2.2 Isolation and Differentiation of Primary Cells

In order to grow ASFV, primary porcine bone marrow cells were harvested from a 6 kg-10 kg juvenile pig. Animals were euthanised using Schedule 1 approved procedure by barbiturate (pentobarbital) overdose. Femur, tibia, ulnar and radius bones were collected and crushed. Crushed bones were washed in phosphate buffered saline (PBS) (The Pirbright Institute Media Services, or Sigma Aldrich) supplemented with PS to a final concentration of 100 U.mL for 2 hours with shaking. Bone fragments were filtered using muslin cloth and remaining supernatant was collected. Solution was centrifuged at $350 \times g$ for 10 minutes and cells washed in PBS, re-spun at the same conditions to isolate cells. Monocyte cells were purified by Ficoll-Paque™Plus Premium Sterile (GE

Healthcare) gradient. Briefly, Ficoll-Paque™ Plus was overlaid with the cell suspension, centrifuged at 1000 x *g* for 20 minutes with no brake, and the buffy coat collected. After washing with PBS, the cells were seeded in Roswell Park Memorial Institute media (RPMI) supplemented with 50 U.mL PS, 10% FCS, 0.4% 1M 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) (Sigma Aldrich) and 100 ng.mL of recombinant porcine macrophage colony-stimulating factor fused to the Fc region of porcine IgG1a (CSF1-fc) (BioRad) at appropriate densities for experiment.

Post-muslin filtered non-purified cells used for viral titrations were plated in complete Earle's Balanced Salt Solution (EBSS) (Gibco) with 10% pig serum (BioSera), 100 U.mL PS, and 0.4% 1M HEPES. Porcine alveolar macrophages were collected from the lungs by broncho-alveolar lavage. Cold Dulbeccos PBS without Mg⁺ Ca⁺ was spun at 470 x *g* for 2 min, washed in PBS and re-spun. Cells were diluted for seeding in RPMI (Corning) supplemented with 10% FCS, and 100 U.mL PS.

2.3 Virus Methods

All virus work was performed under Specified Animal Pathogen Order 4 (SAPO4) conditions at The Pirbright Institute high containment laboratories.

2.3.1 Propagation of Viral Stocks

ASFV strains were propagated in porcine bone marrow cells seeded in T175 flasks (See 2.2.2). Cells were cultured at 37°C, supplemented with 5% CO₂ and

95% humidity. Cells were infected with virus (Georgia07/1, GeorgiaΔMGF, OURT88/3). After 4 days cells were inspected for the presence of rosettes or cellular detachment. Media was removed and centrifuged at 500 x g for 10 minutes. Supernatant was divided in to 1 mL aliquots and stored at -80°C. Stocks of GeorgiaΔMGFAΔK145R and GeorgiaΔMGFBΔK145R isolates were gifted from Anusyah Rathakrishnan.

2.3.2 TCID₅₀ Viral Titration

In order to titrate the virus for Median Tissue Culture Infectious Dose (TCID₅₀) of viral stocks, primary porcine bone macrophage cells were seeded in 96 well plates at a density of $1-1.6 \times 10^6$ cells per well and incubated at 37°C with 5% CO₂ for 72 hours in complete EBSS medium (10% pig serum, 100 U.mL PS, 0.4% 1M HEPES). Plates were shaken for 15 seconds and supernatant discarded. 100 µL of viral stock was used for serial dilution (10^{-1} – 10^{-12}) performed in quadruplicate. The serial dilutions were transferred to the cell plates. Plates were sealed and cells were further incubated at 37°C with 5% CO₂ for 72 hours.

Cells were fixed in 4% paraformaldehyde for 1 hour, and permeabilised with PBS 0.2% Triton X-100 (Sigma Aldrich) for 5 minutes at room temperature. Cells were incubated in mouse anti-p30 antibody produced in house (1:1000 in PBS 1% bovine serum albumin (BSA) (Sigma Aldrich) for 1 hour. Cells were incubated in secondary anti-mouse Alexa Fluor 488 (Invitrogen) (1:500 in 1% BSA/PBS) for 1 hour. Cells were washed in PBS three times between each step. Work was

performed at room temperature, and incubation periods were in the dark post addition of fluorescent antibody. Finally, cells were stored in PBS and observed using a fluorescent microscope. Positive wells were counted and TCID₅₀ calculated with Spearman and Käber algorithm (Hierholzer and Killington 1996).

2.4 Plasmids and Nucleic Acid Techniques

2.4.1 RNA Extraction

RNA was extracted from cells using RNeasy extraction kit (QIAGEN) following the manufacturer's protocol. Cells were lysed in lysis buffer, and lysate further disrupted by using a 25G needle. The RNA was purified using RNeasy Mini silica-membrane spin columns to remove gDNA and eluted with 50 µL RNase/DNase free water. The amount of RNA was quantified using a NanoDrop™ One spectrophotometer.

2.4.2 Viral DNA Extraction

Viral DNA was extracted from infected cells using QIAamp Viral RNA Mini Kit (QIAGEN). The manufacturer's protocol was followed as described. Cell culture was incubated in 140 µL of AVL buffer with carrier RNA for 10 minutes to ensure virus particle lysis. Samples were mixed with absolute ethanol, and DNA purified using a QIAamp Mini column by centrifugation. Viral DNA was washed in AW1 and AW2 buffer through spin columns. RNA was eluted in 60 µL AVE buffer. The amount of viral DNA was quantified using a NanoDrop™ One spectrophotometer.

2.4.3 cDNA Synthesis

cDNA was formed from extracted cellular RNA using Superscript™ III First-Strand Synthesis System (Invitrogen) to manufacturer's guidelines. Amount of genetic material used per sample depended on availability (85-726 ng) determined by NanoDrop™ One spectrophotometer. Samples were incubated in 50 µM of Oligo d(T)₂₀ primers, 10mM dNTP mix, and nuclease free water to a total volume of 10 µL. Reactions were mixed, heat treated at 65°C for 5 minutes, and kept on ice for at least 1 minute. Reactions were further incubated in 2 µL of 10x reverse transcriptase buffer, 4 µL of 25mM MgCl₂, 2 µL of 100 mM Dithiothreitol (DTT), 1 µL RNaseOUT™, and 1 µL of Superscript™ III reverse transcriptase (200 U.µL). Reactions were incubated at 50°C for 10 minutes, and then reactions terminated at 85°C for 5 minutes. Finally, reactions were incubated in 1 µL of *E. coli* RNase H and incubated at 37°C for 20 minutes. Samples were stored at -20°C.

2.4.4 Plasmids and Cloning

2.4.5 Vectors

Constructs containing host genes for Yeast-2-Hybrid screening in the pGADT7 or pHON3 vectors were a kind gift from Steve Goodbourn (St. Georges University of London, UK) (See Table 4-2). Similarly, Gal4-luciferase reporters (pGal4.Luc) and stimulus constructs (p.Gal4-p65, p.Gal4.IRF3, p.Gal4-147) were also generously supplied by Steve Goodbourn. pJAC.LacZ was a kind gift from Michael Baron (The Pirbright Institute, UK). Constructs containing host genes for expression in

luciferase assay systems in various vectors, and p.MC160.HA were a kind gift from Joanna Shisler (University of Illinois, USA). pRK5.flag.TRAF6 was a generous gift from Andrew Bowie (Trinity College Dublin, Republic of Ireland). pCMV5.p300.HA was a generous gift from Tony Kouzarides (University of Cambridge). p.Npro.GFP was kindly supplied by Julian Seago (The Pirbright Institute, UK). Firefly luciferase under the control of NFκB and IRF3 regulatory elements (p.NFκB.Luc, p.IRF3.Luc) and renilla luciferase (p.TK.Renilla) were also from Joanna Shisler. pcDNA3.1 vector containing Cul2.Myc, Cul5.Myc, T7.EloC, and HA.EloB were purchased from Addgene Plasmid Repository (Addgene).

ASFV genes for Yeast-2-hybrid screening were cloned into pGBKT7 vector. Porcine STING and porcine cGAS for Yeast-2-hybrid were cloned into pGADT7. Constructs expressing MGF360-10L, 11L, 12L, 13L, 14L, MGF505-1R, 2R, and 3R for use in mammalian expression systems were designed based on the sequence from the Georgia07/1 isolate (GenBank: FR682468.2) and synthesised by GeneArt® into pcDNA3.1 vector via *Bam*HI and *Eco*RI restriction sites, and to include fusion to a C-terminus V5 (GKPIPPELLGLDST) tag. Constructs were codon optimised for expression in human cells.

pUC57 plasmids containing MGFA and MGFB inserts to be used in homologous recombination for generation of virus gene deletion mutants were designed using SnapGene software and synthesised by Twist Bioscience.

2.4.6 General Cloning Techniques

2.4.6.1 Polymerase Chain Reaction Amplification of Target Inserts

Plasmid constructs expressing genes of interest were produced by cloning relevant polymerase chain reaction (PCR) fragments amplified from virus genome or porcine macrophage cDNA. Forward and reverse primers were designed for the gene of interest (porcine STING, porcine cGAS, MGF360-10L, MGF360-11L, MGF360-12L, MGF360-13L, MGF360-14L, MGF505-1R, MGF505-2R, and MGF505-3R). Primers were designed to include desired suitable restriction enzyme sites for cloning (See Table 2-2). All primers were synthesised by Sigma Aldrich and purified by desalt purification. RNA was extracted from primary porcine alveolar macrophages (See 2.2.2) infected with attenuated ASFV isolate OURT88/3, a known inducer of type-I interferon, in order to induce the expression of interferon stimulated genes porcine STING and porcine cGAS from which cDNA was reversed transcribed. For amplification of viral genes viral DNA was extracted from porcine macrophages infected with Georgia07/1; PCR reactions were performed using AccuPrime PFX Supermix (Invitrogen) (22.5 µL), forward and reverse primers (200nM at final concentration, 0.5 µL), and sample DNA (1.5 µL). The PCR reaction was performed at 95°C for 5 minutes, then 40 cycles of 95°C for 15sec, 55-65°C for 30secs (annealing temperature depended on individual primers), 68°C for 150secs. The expected size of PCR products was confirmed by agarose gel electrophoresis (See 2.4.6.2), or stored at -20°C.

Primer	Features	Sequence
Po-cGAS - Forward	<i>NdeI</i>	GGTACC CATATG ATGGCGGCCCGGCGGGGAAAGTCCACGC
Po-cGAS - Reverse	<i>BamHI</i>	GGTAC GGATCC TCACCAAAAACTGGAAATCCATTGT
Po-STING – Forward	<i>NdeI</i>	GATATAA CATATG ATGCCCTACTCCAGCCTGCATCCATC CATC
Po-STING - Reverse	<i>EcoRI</i>	GGTAC GAATTC TCAGAAGATATCTGAGCGGAGT
MGF360-10L - Forward	<i>EcoRI</i>	GGTAC GAATTC ATGTTTCCTTCCTTACAATCC
MGF360-10L - Reverse	<i>BamHI</i>	GGTAC GGATCC CTAGGCCAACATACTTTTAAATTTATAG
MGF360-11L – Forward	<i>EcoRI</i>	GGTAC GAATTC ATGTTGCCTTCCCTGCAATCCCTCACGA AGAAAGTACTGGC
MGF360-11L - Reverse	<i>BamHI</i>	GGTAC GGATCC TTATCTTTGTTTATAATCAAGAAAAATC CC
MGF360-12L – Forward	<i>EcoRI</i>	GGTAC GAATTC ATGTTGCCTTCCCTGCAATCTCTGACCA AAAAGGTGTTGGC
MGF360-12L - Reverse	<i>BamHI</i>	GGTAC GGATCC TCATCTTAAATCATAGG
MGF360-13L – Forward	<i>EcoRI</i>	GGTAC GAATTC ATGTCGTTGCCGCTCTCTCTGC
MGF360-13L - Reverse	<i>BamHI</i>	GGTAC GGATCC CTATAGTATATTATGAGAATATTCC
MGF360-14L – Forward	<i>EcoRI</i>	GGTAC GAATTC ATGTTGTCTTTACAAACGTTGGCC
MGF360-14L - Reverse	<i>BamHI</i>	GGTAC GGATCC TTAGTCTATAACTATAATTTCTGGATGG GC
MGF505-1R – Forward	<i>EcoRI</i>	GGTAC GAATTC ATGTTCTCTCTCCAGAACTTATGTCTG
MGF505-1R - Reverse	<i>BamHI</i>	GGTAC GGATCC CTAAGGCATGTATTGTTTAGAATAAATG G
MGF505-2R – Forward	<i>EcoRI</i>	GGTAC GAATTC ATGTTTTCCCTTCAAGACCTTTGCCGAA AGCATC
MGF505-2R - Reverse	<i>BamHI</i>	GGTAC GGATCC TTAACATTGTATTGTTTTATAATGAATT AC
MGF505-3R – Forward	<i>EcoRI</i>	GGTAC GAATTC ATGTCCTCTTCTCTTCAGGAACCTTGTC G
MGF505-3R - Reverse	<i>BamHI</i>	GGTAC GGATCC CTAGCTTTCCAGGTTTCGAAG
MGF360-12L.TRAF6.V5 Forward	Used for SDM	ATCTTCGCGGAGCTGAGAGAGCTGATC
MGF360-12L.TRAF6.V5 Reverse	Used for SDM	CGCGATCGCCATCTTGATGTTGTTCACTTTC

Table 2-2. Primer sequences used for gene amplification for cloning

2.4.6.2 Agarose Gel Electrophoresis

Samples were run on 1% agarose gels in 1x TAE with 0.04% ethidium bromide (Sigma Aldrich) run at 100V for 1 hour and imaged under UV light by using a Biorad Transilluminator and Quantity One version 4.1.0 software (Biorad). Successful amplification products were excised using a clean scalpel. DNA was extracted using GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) following manufacturer's protocol.

2.4.6.3 Restriction Enzyme Digestion of DNA

Vector DNA and insert amplified PCR product to be used in cloning were digested by appropriate restriction enzymes. For cloning MGF360-10L, 11L, 12L, 13L, 14L, MGF505-1R, 2R, 3R into pGBKT7, *EcoRI*-HF (New England Biolabs) and *Bam*HI (Promega or New England Biolabs) enzymes were used. Whereas po-STING, and po-cGAS into pGADT7 were digested with *Nde*I (New England Biolabs) and *EcoRI*-HF, and *Nde*I and *Bam*HI respectively. Restriction digests of inserts were carried out in 30 µL reactions according to specification of required enzymes, 26 µL of insert of interest PCR product, 0.5 µL of each enzyme and 3 µL of appropriate buffer (Buffer E (Promega), or CUTSMART (New England Biolabs)). Digestion of vector was performed in 50 µL reactions. 10 µg of plasmid DNA was digested with 5 µL of appropriate enzymes, in 5 µL of appropriate buffer, remaining volumes was made up with RNase free water. Reactions were incubated at 37°C for 2 hours, and heat inactivated at 65°C for 20 minutes. Digests were stored at -20°C.

2.4.6.4 Ligations

Ligations were set up as 20 µL reactions using 1 µL of T4 DNA ligase (Promega), with 10 µL 2x buffer (Promega), and varying volumes of plasmid and insert. For ligation of MGF genes to pGBKT7 a vector:insert molar ratio of 1:3 was used. (Amount of insert varied between 35 ng – 44 ng), whereas for ligation of po-STING and po-cGAS to pGADT7 the molar ratio was 1:4 (amount of insert was 77 ng – 119 ng respectively). The reaction was incubated at room temperature for a minimum of 5 minutes. Ligation reactions were then transformed into chemically competent *E. Coli* (See 2.4.8). Successful ligation was determined by restriction enzyme digestion agarose gel electrophoresis (See 2.4.6.3) and Sanger sequencing (See 2.4.9).

2.4.7 Site Directed Mutagenesis

Plasmid constructs containing mutant variants of ASFV genes (MGF360-12L.mVHL.V5, MGF360-12L.mBC.V5, MGF360-12L.mCUL.V5, MGF505-1R.mSOCS.V5, MGF505-1R.mBC.V5, MGF505-1R.mCUL.V5) in pcDNA3.1 vector were synthesised by GenScript.

pcDNA3.1.MGF360-12L.TRAF6.V5 was created from pcDNA3.1.MGF360-12L.V5 using Q5® Site-Directed Mutagenesis Kit (New England Biolabs) according to the manufacturer's protocol. Mutagenesis primers were designed using NEB Base Changer and Agilent Primer Design online software and synthesised by Sigma Aldrich Custom Oligos followed by desalt purification (See Table 2-2). Competent

DH5α *E. coli* were transformed with the resulting product, and selectively grown on agar media containing ampicillin corresponding to pcDNA3.1 vector antibiotic resistance (See 2.4.8). Individual colonies were amplified and DNA purified by miniprep (See 2.4.8). Successful substitution of the target residues was determined by Sanger Sequencing (See 2.4.9).

2.4.8 Transformation Of *E. Coli* and Plasmid Scale Up

Chemically competent DH5α *E. Coli* were transformed with target plasmid for scale up. 50 µL of *E. Coli* were incubated with 1.0 µL of plasmid or ligation reaction on ice for 15 minutes and heat shocked at 42°C for 1min. Transformed *E. Coli* were incubated in lysogeny broth (LB) growth media (Gibco) for 1 hour at 37°C with shaking. Bacteria were plated on to Agar plates with appropriate antibiotic selective marker, ampicillin (100 µg.mL) (Sigma Aldrich, A9518) or kanamycin (50 µg.ml) (Sigma Aldrich). Plates were left at 37°C overnight. Individual colonies were selected and taken forward for plasmid purification by either minipreps (small scale up) or midiprep (large scale up). Minipreps and midipreps were performed using QIAGEN® Mini (QIAGEN) or Midi kits (QIAGEN) according to the manufacturer's instructions. For minipreps, single bacterial colonies were grown overnight in 5 mL LB broth supplemented with appropriate antibiotic with shaking (200 RPM). Bacteria were pelleted and lysed, DNA was isolated using QIAprep2.0 spin columns and eluted in 50 µL RNase/DNase free water. For midipreps, single bacterial colonies were grown overnight in 100 mL LB broth supplemented with appropriate antibiotic with shaking (200 RPM). Bacteria were pelleted by centrifugation (4500 x *g* for 15 minutes at 4°C) and

lysed, DNA was isolated using QIAGEN-tip columns via gravity flow. DNA was precipitated with isopropanol by centrifugation (20000 x g for 45 minutes at 4°C), washed in 70% ethanol, and resuspended in 200 µL RNase/DNase free water. The concentrations of purified plasmids were measured using NanoDrop™ One spectrophotometer.

2.4.9 Sanger Sequencing

To confirm the correct insert, plasmids were prepared by Miniprep or Midiprep (See 2.4.8) and sequenced by Sanger Sequencing. Approximately 500 ng of plasmid was diluted in 10 µL RNase and DNase free water and heated to 96°C for 1 minute to denature the plasmid. To form PCR products BigDye™ Terminator v3.1 Sequencing Kit (Applied Biosystems) was used. 1.0 µL of target plasmid was incubated with: 5x sequencing buffer (2.0 µL), BigDye™ Terminator v3.1 (0.5 µL), primers 1.6 pmol (3.0 µL) and RNase/DNase free water (3.5 µL). PCR was run at the following conditions in Table 2-3. PCR. PCR products were subsequently ethanol precipitated. 125mM EDTA (5 µL) and 100% ethanol (60 µL) were added to PCR product reaction. Samples were incubated at 15 minutes in the dark to precipitate extension products and recovered by centrifugation at 890 x g for 45 minutes. The supernatant was removed by inverse pulse spinning (plate facing downwards) at 7 x g. PCR products were washed in 70% ethanol, and further spun at 600 x g for 15 minutes, and inverse pulse-spun to remove the supernatant. PCR product pellets were air dried at room temperature.

Sanger Sequencing was performed at The Pirbright Institute by Jemma Wadsworth. Results were analysed using VectorNTI Advance® or SnapGene Software.

Temperature (°C)	Time	No. of Cycles
96	1 min	1
96	10 sec	
50	5 sec	25
60	4 min	
10	hold	

Table 2-3. PCR conditions

2.5 Yeast-2-Hybrid

2.5.1 Transfection of Yeast Cells

Genes of interest were cloned into plasmid vectors, or generously gifted from Steve Goodbourn (St George's University). Gene cloned into pGBKT7 and pGADT7 were under the control of the T7 promoter for *in vitro* expression. Cloning of genes adjacent to the Gal4 DNA Binding Domain (pGBKT7) or Gal4 Activation Domain (pGADT7) resulted in the expression of gene of interest fused with respective domains at the N-terminus. Expression of fusion proteins was driven by the constitutively active ADH1 promoter. Plasmids contained genes encoding for selectable markers TRP1 and LEU2 allowing for selection of successful transfectants on appropriate amino acid dropout media, Tryptophan or Leucine negative media.

Transfection was performed using the Lithium Acetate (LiAc)-mediated method (Takara). To scale up competent yeast cells, colonies were picked and grown in 50ml yeast extract peptone dextrose growth media (YPD) (Takara) at 30°C with 250 RPM agitation for 16-18hours until in stationary growth phase (Optical density at 600nm (OD₆₀₀) >1.5). Cultures were transferred to 300ml YPD media and incubated up to 24hours until in the log growth phase (OD₆₀₀ 0.4-0.6). Optical density was measured with Ultrospec 2100 Pro UV/visible spectrophotometer (Biochrom). Yeast cells were harvested by centrifugation (1000 x *g* for 5 minutes at room temperature) and re-suspended in 1X TRIS-EDTA Buffer (TE)/1X LiAc, mixed with 0.1ug of plasmid DNA and 0.1mg of carrier DNA

and sterile polyethylene glycol (PEG)/LiAc. Yeast were further incubated for 30 minutes. Dimethyl sulphoxide (DMSO) (Sigma Aldrich) was added to the cells before being heat shocked at 42°C for 15 minutes and chilled on ice for 2 minutes. Cells were isolated by centrifugation and re-suspended in 1xTE buffer then spread onto agar plates containing the appropriate selection medium and incubated at 30°C upside down until the appearance of yeast colonies, typically 2-4 days. Colonies were picked and re-streaked on relevant dropout media plate and stored at 4°C. Colonies were picked and suspended in 25% glycerol yeast extract peptone dextrose adenine (YPDA) solution (Takara) and stored at -80°C.

2.5.2 Selection of Optimal Growth Conditions for Yeast

Transfected yeast were tested for potential self-activation via leaky expression of reporter gene HIS3 (See 4.2.2 for further details on self-activation), by growth on -His dropout media in presence of increasing concentration of 3-amino-1,2,4-triazole (3AT) (0-50 mM) (Merck), a competitive inhibitor of HIS3 protein used to control unwanted expression. Leaky HIS3 expression could lead to the growth of false positive colonies. Growth on this single dropout media in the absence of 3AT indicates leaky HIS3 expression. The lowest concentration of 3AT that eliminates any colony growth was used in further downstream applications to prevent growth of false positive colonies for the relevant transfected yeast.

2.5.3 Mating of Transfected Yeast Strains

As I intended to investigate potential interactors of 8 ASFV MGF genes and 18 genes (See Table 4-1 and Table 4-2) of the innate immune system, sequential transfections would have resulted in over 200 individual transfections. Thus, in order to preserve time and consumables a mating approach was performed (Bendixen, Gangloff *et al.* 1994). pGBKT7 and pGADT7 plasmids containing genes of interest were transfected into yeast strains of opposite sex (Y187 Mat-a and AH109 Mat-a), this allows a convenient method of generating yeast containing both plasmids. Successfully transfected yeast were picked from dropout media and incubated in YPDA at 30°C with 250 RPM agitation overnight. Successful mating was initially determined by 0.4% Trypan blue (Gibco) staining and the observation of zygotes with typical 3 lobed “Mickey Mouse”-like structures observed under phase microscopy. Mated yeast were then grown on double dropout media (-Trp, -Leu) agar plates for selection of yeast containing both plasmids at 30°C for 2-4 days. Mated colonies were picked and suspended in 25% glycerol YPDA solution and stored at -80°C.

2.5.4 Yeast-2-Hybrid Interaction Assays

Yeast successfully mated to contain a pGBKT7 plasmid with one of the ASFV-MGF360-10L, 11L, 12L, 13L, 14L, MGF505-1R, 2R, 3R genes inserted, and pGADT7 plasmid with a gene encoding for a component of the innate immune system, were streaked on quadruple dropout media (-Tryptophan (Trp), -Leucine (Leu), -Histidine (His), -Adenine (Ade)) and an appropriate concentration 3AT and incubated upside down at 30°C for up to 10 days. Colonies were picked and re-

streaked on to quadruple dropout media, with appropriate an 3AT concentration and containing X- α -Gal (Sigma Aldrich) (20mg.ml) at 30°C for 48hours, for colorimetric quantitation of the expression of positive interaction reporter gene MEL1. Colonies expressing MEL1 and thus α -Galactosidase catalyses the chromogenic X- α -Gal substrate, turning the colonies blue and acting as additional and stringent confirmation of positive interaction. Successful interactions were photographed.

2.6 Luciferase Assays

2.6.1 Luciferase Assay - Cell Preparation

HEK293T cells were seeded in 24 well plates (Corning) at a density of 1.25×10^5 cells per well. Cells were seeded in DMEM Glutamax with 10% FCS and 1% PS. Cells were incubated at 37°C for 24 hours before transfection.

2.6.2 Luciferase Assay – Transfection

HEK293T cells were observed under light microscope to ensure a confluency of at least 60%. Cells were transfected with plasmids using TransIT-LT1 (Mirus Bio). All cells were transfected with firefly luciferase reporter plasmids including pNFkB.Luc (100 ng.well), or pIRF3.Luc (100 ng.well), and an endogenous control plasmids pTK.Renilla (10 ng.well), or p.LacZ (25 ng.well). Cells were co-transfected with either the empty vector (pcDNA3.1) or vector expressing viral proteins at 350 ng.well, and exogenously expressed stimulant if used (See 2.6.3).

To ensure consistency across all condition's total DNA per well was equalised with empty vector. Each technical experiment contained biological duplicates of unstimulated and stimulated conditions.

2.6.3 Luciferase Assay – Stimulation of Cells

HEK293T cells were stimulated by transfection with plasmids expressing various proteins (IKK α .flag, IKK β .flag, flag.TBK1, IRF3-5D, or Gal4-IRF3-65-427 at 100 ng.well; or p65-GFP or Gal4DBD-p65 at 5 ng.well) and incubated for 24 hours; or using various stimulants TNF α (20 ng. μ L), IL-1 β (10 ng. μ L), or Sendai virus Cantell strain (200HAU.well) (Charles River). Stimulants TNF α and IL-1 β were left for 6 hours, or Sendai virus for 24 hours, before cell lysis. Any discrepancy between the amount of DNA to be transfected in stimulated and unstimulated conditions was made up with empty vector pcDNA3.1.

2.6.4 Dual Luciferase Assay – Cell Lysis and Luciferase Reading

After stimulation, media was aspirated, and cells lysed in 100 μ L 1X Passive Lysis Buffer (Promega) for 15 minutes with shaking followed by freeze thawing at -20°C. 20 μ L of each cell lysate was added to a well of a 96 well plate. Dual-Luciferase reagents (Luciferase Buffer II and Stop-Glo buffer) were used to measure luciferase activity (Promega). Using a Cytation 3 dispenser plate reader (BioTek) 100 μ L of Luciferase Buffer II solution was added to a well, after 2 second delay firefly luciferase expression was read at a gain of 175 and 10 second integration time. To read renilla luciferase output, 100 μ L of Stop-Glo

buffer was added to the same well, following a 2 second delay, output was measured at 175 gain and 10 second integration time.

2.6.5 β Galactosidase Assay – Cell Lysis and Reading

HEK293T cells transfected with luciferase reporter plasmid and endogenous control p.LacZ expressing β galactosidase (β gal) were lysed in 100 μ L 1X Reporter Lysis Buffer (Promega) for 15 minutes with shaking then freeze thawed at -20°C. 20 μ L of lysate was added per well in a 96 well plate and firefly luciferase expression was measured using a Cytation 3 dispenser plate reader (BioTek). 100 μ L Luciferase assay Buffer I (Promega) was added to individual wells, after a 2 second delay luciferase readout was measured at 175 gain and 10 second integration time. Each well was read consecutively.

To measure control β Galactosidase activity, 10 μ L of lysate corresponding to cells from an individual well of a 24 well plate, was added to a well of a 96 well plate, then 70 μ L of GalactonPlus (1:100 dilution) (Applied Biosystems) was added to each well. The plate was incubated at room temperature in the dark for 40 minutes and subsequently read on Cytation 3 dispenser plate reader (BioTek). 100 μ L of Accelerator-II buffer (Applied Biosystems) was added to individual well and after a delay of 2 seconds, β Galactosidase activity was measured at 175 gain and 0.5 second integration time. Each well was then measured in a consecutive manner.

2.7 Protein Characterisation

2.7.1 SDS-PAGE – Western Blotting Sample Preparation

HEK293T cells grown in 6 well plates (Corning) were washed three times with PBS and lysed with 100ul.well 1x Radioimmunoprecipitation assay (RIPA) buffer (ThermoFisher) containing 1x Halt Protease and Phosphatase Cocktail inhibitor with EDTA (ThermoFisher) and incubated for 10 minutes. Samples were spun down at 20000 x *g* to remove debris. Cells were lysed on ice, with ice cold reagents. Samples for sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE) were diluted to 1x in Pierce™ Lane Marker Reducing Sample Buffer (ThermoFisher) containing DTT as reducing agent. Prior to gel loading, samples were incubated at room temperature for 30 minutes. This was instead of the more typical 95-100°C for 5 minutes as in these conditions MGF genes appeared to aggregate and would not pass into the resolving gel.

2.7.2 SDS-PAGE – Co-Immunoprecipitation

2.7.2.1 Antibody Coupling to Magnetic Epoxy M-270 Beads

1mg of magnetic Epoxy M-270 Dynabeads™ were covalently coupled to 5 µg of V5-tag antibody by rotation on windmill rotor (Agar Scientific) at room temperature overnight. Dynabeads™ Co-Immunoprecipitation (CoIP) Kit (ThermoFisher) protocol was used according to the manufacturer's protocol. The magnetic beads coupled to antibodies were washed in HB and LB buffers supplemented with 0.05% Tween-20 (Sigma Aldrich) and collected using a

magnet. Coupled beads were stored in SB buffer at 4°C at a concentration of 10mg.ml.

2.7.2.2 ColP – Sample Preparation

HEK293T cells were seeded in 6 well plates at 6.25×10^5 cells per well and transfected with TransIT-LT1 (MirusBio). After 24 hours cells were washed three times in ice cold PBS. If noted in text, cells were incubated in 2 mL of cross-linking agent dithiobis(succinimidyl propionate) (DSP) (ThermoFisher) dissolved in anhydrous DMSO (Sigma Aldrich) to a final concentration of 2 mM in PBS on ice for 2 hours, and 1M Tris HCl at pH 7.5 (Invitrogen) was added at a final concentration of 20mM for 15 minutes to quench the reaction. Cells were subsequently washed three times in ice cold PBS and incubated in 200 μ L 1x IP buffer supplemented with 1x Halt Protease and Phosphatase Cocktail inhibitor without EDTA (extraction buffer) for 15 minutes before being scraped off. Samples were spun down at 20000 x g at 4°C for 10 minutes. 10% of the sample supernatant was retained as whole cell lysate input, and the remaining lysate was used for ColP using the ThermoFisher Epoxy270 Dynabead ColP Kit protocol to manufacturer's instructions. All steps were performed on ice.

Samples for ColP were incubated with 1.5 mg magnetic Epoxy270 Dynabeads™ previously covalently coupled to antibody and washed in extraction buffer, on a windmill rotor (Agar Scientific) at 4°C for 45 minutes. Samples were then washed three times in extraction buffer, and once in last wash buffer (LWB). Samples incubated with magnetic beads were finally resuspended in 60 μ L Elution Buffer.

After elution, samples for SDS-PAGE were diluted to 1x in Pierce™ Lane Marker Reducing Sample Buffer (ThermoFisher) containing DTT as reducing agent and incubated at 100°C for 5 minutes.

2.7.3 SDS-PAGE – Gel Electrophoresis

PreCast Mini-Protean™ TGX™ 4-20% (BioRad) or homemade gels of polyacrylamide (Sigma Aldrich) concentration between 8-12%, were used for electrophoresis of proteins. 10 µL of reduced sample was loaded and the gel run at 200V for 35 minutes in 1x TRIS-Glycine - SDS (TGS-SDS) (BioRad). Protein ladders Full Range Rainbow Ladder (GE Healthcare), or Precision Plus Protein™ Dual Color Standard were used (BioRad).

2.7.4 Protein Transfer to PVDF Membranes

Proteins were transferred to methanol activated Polyvinylidene difluoride (PVDF) membranes (pore size of 0.2 µm or 0.4 µm) (GE Healthcare) using Mini Trans-Blot® Electrophoretic Transfer Cell (BioRad) in ice cold 1x TGS-SDS with 20% methanol at 100V for 90 minutes.

2.7.5 Membrane Blotting

Membranes were blocked in 5% Milk TRIS-Buffered Saline Tween (TBST) at room temperature for 1hr and incubated in primary antibody diluted in 5% BSA TBST or 5% Milk TBST (See Table 2-4.) overnight at 4°C with rolling. Three washes of 5

minutes in TBST were performed between each step. Membranes were incubated in species appropriate HRP conjugated secondary antibody diluted in 5% Milk TBST for 1-2hrs at RT. Membranes were then incubated in Pierce™ ECL Plus Western Blotting Substrate (ThermoFisher) for 4 minutes before being imaged on SynGene G:Box. Images were processed using PowerPoint 2011 (Microsoft) or Illustrator CS6 software (Adobe). ImageJ (NIH) software was used to quantify western blots where applicable.

Antibody Target	Species	Dilution	Company
Anti-Mouse IgG (H+L) HRP	Goat	1:1000	Invitrogen
Anti-Rabbit IgG HRP	Mouse	1:2000	Cell Signalling
Anti-Rat HRP	Goat	1:500	DAKO
CBP (D6C5)	Rabbit	1:1000	Cell Signalling
Cullin-2	Rabbit	1:250	Invitrogen
Cullin-5 (EPR14725)	Rabbit	1:1000	AbCam
Elongin B (EPR10441)	Rabbit	1:1000	AbCam
Elongin C	Rabbit	1:1000	AbCam
Flag-Tag	Rabbit	1:1000	Cell Signalling
Flag-Tag M2	Mouse	1:1000	Sigma
GFP-Tag (FL) sc-8334	Rabbit	1:500	Santa Cruz
GFP-Tag (A11121)	Mouse	1:1000	Invitrogen
HA-Tag	Rat	1:1000	Roche
IKK α (3G12)	Mouse	1:1000	Cell Signalling
IKK β (D30C6)	Rabbit	1:1000	Cell Signalling
IRF3 (D83B9)	Rabbit	1:1000	Cell Signalling
Myc-Tag (9B11)	Mouse	1:1000	Cell Signalling
p300 (D8Z4)	Rabbit	1:1000	Cell Signalling
p65 (D14E12) XP®	Rabbit	1:1000	Cell Signalling
Phospho-p65 (S536) (93H1)	Rabbit	1:1000	Cell Signalling
Rpb1 NTD (D8L4Y)	Rabbit	1:1000	Cell Signalling
T7-Tag (D9E1X) XP®	Rabbit	1:1000	Cell Signalling
TBK1 (D1B4)	Rabbit	1:1000	Cell Signalling
TFIIB (2F6A3H4)	Mouse	1:1000	Cell Signalling
TRAF6 (D21G3)	Rabbit	1:1000	Cell Signalling
V5-Tag	Mouse	1:1000	BioRad
V5-Tag HRP	Rabbit	1:2000	Bethly Inc.
α -Tubulin (DM1A)	Mouse	1:1000	Cell Signalling
β 3-Tubulin	Mouse	1:1000	Invitrogen

Table 2-4. Antibodies used for western blotting and CoIP

2.8 Immunofluorescent Microscopy

2.8.1 Immunofluorescent Staining

HeLa or A549 cells were seeded on sterile glass coverslips (SLS) in 24 well plates at density of 5×10^4 cells per well in DMEM GlutaMax supplemented with 10% FCS, and 1% PS for 24 hours at 37°C. Cells were transfected with relevant plasmids of interest typically at 450 ng.well using TransIT® LT1 (Mirus Bio), and further incubated for 24 hours. HeLa cells were subsequently stimulated with TNF α (20 ng.mL) for 30 minutes, or A549 cells with Sendai virus (200 HAU.mL) for 4 hours. Cells were washed three times in PBS before fixation and between each subsequent step. After fixation in 4% paraformaldehyde (ChemCruz, sc-281692) in PBS for 20 minutes at room temperature, cells were incubated for 10 minutes with permeabilising agent 0.2% Triton X-100. Permeabilized cells were then blocked for 1 hour at room temperature with 2% BSA in PBS. Cells were incubated in 200 μ L of 0.2% BSA in PBS with primary antibodies for 1 hour followed by incubation with 200 μ L secondary antibodies in 0.2% BSA for 1 hour at room temperature in the dark. Finally, cells were stained in 4',6-diamidino-2-phenylindole (DAPI) (1:10000) for 10 minutes and coverslips mounted to glass slides using Vectashield® mounting media (Vector Laboratories). Cells were visualised using a Leica TCS SP8 confocal microscope (Leica) and processed with LASX or LAS AF Lite confocal software (Leica).

	Antibody	Species	Dilution	Company
Primary Antibodies	Anti-IRF3 Polyclonal (1HCLC)	Rabbit	1:100	Invitrogen
	Anti-V5-Tag IgG2a	Mouse	1:350	BioRad
	Anti-p65 D14E12 XP®	Rabbit	1:400	Cell Signalling
Secondary Antibodies	Alexa Fluor Anti-Mouse 568	Goat	1:500	Life Technology
	Anti-Rabbit IgG (H+L) Superclonal Recombinant Alexa Fluor 488	Goat	1:2000	Invitrogen
	Anti-Rabbit Alexa Fluor 488	Goat	1:500	Life Technology

Table 2-5. Antibodies used in immunofluorescent staining

2.9 qPCR Analysis

2.9.1 Infection of Porcine Bone Marrow Cells for qPCR Gene Expression Analysis

Gene expression changes were measured by quantitative real time PCR (qPCR). Porcine bone marrow cells were seeded in 24 well plates at density of 1×10^6 cells per well in 500 μ L of complete RPMI and 100 ng/mL of porcine CSF1-fc (BioRad) and incubated at 37°C and 5% CO₂ for 48 hours. Media was removed and 500 μ L of previously titrated and diluted viral stocks of OURT88/3, Georgia07/1, Georgia Δ MGFA Δ K145R or Georgia Δ MGFB Δ K145R were added to wells at a multiplicity of infection (MOI) of 2. The experiment was performed in duplicate. The supernatant was removed after 0hr, 2hr, 8hr, 16hr and cells lysed for RNA extraction (See 2.4.1) and stored at -80°C, until used for subsequent cDNA synthesis (See 2.4.3).

2.9.2 Removal from SAP04

qPCR was carried out in Zoetis Inc. laboratories in Olot, Spain, therefore cDNA samples required treatment to enable their removal from The Pirbright Institute's SAP04 high containment facilities. Samples were added to screw cap 2 mL tubes and incubated with 10% of volume sodium acetate pH ≤ 5.6 and 2.5x total volume 100% ethanol added. Samples were heated at 56°C for 2 hours. Tubes containing samples were then disinfected and removed from the laboratory, and later couriered to Spain in dry ice.

Samples were spun at 20000 x *g* for 35 minutes at 4°C to precipitate the cDNA and the cDNA pellet was washed twice in 70% ethanol. The cDNA pellet was air dried for 20 minutes and resuspended in AE Buffer (QIAGEN) ready for downstream application.

2.9.3 qPCR

All qPCRs were run on Applied Biosystem™ 7500 Fast Real-Time PCR machine. qPCR reactions were setup on ice unless indicated otherwise. 20 µL qPCR reaction master mix was prepared in 2 mL tubes. For 1 reaction master mix included 10 µL of Power UP SYBR Green Master Mix X2 (Applied Biosystems), 0.5 µL of forward and reverse primers at 500 nM (primers were exon spanning, or exon-exon spanning to limit impact of gDNA) (See Table 2-7), and 8 µL of H₂O. 19 µL of master mix were added to individual wells of a 96 well plate (Axygen). 1 µL of sample was added per well, briefly vortexed and plate was sealed with ABI MicroAmp Seal (Applied Biosystems). The plate was spun down using an Eppendorf 5430R centrifuge at 8°C for 2 minutes. qPCR was run with the following programme and melt curve (Table 2-6). Samples were run in duplicate. qPCR analysis was performed using ABI 7500 Real-Time PCR Software v2.3. $\Delta\Delta C_t$ analysis relative to endogenous control Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was performed to assess expression changes.

qPCR Programme			
STEP	Temperature °C	Duration	Cycles
UDG Activation	50	2 mins	1
Dual Lock DNA Polymerase	95	2 mins	1
Denature	95	15 sec	40
Anneal/Extend	60	1 min	
Melt Curve			
STEP	Temperature °C	Duration	Notes
1	95	15 sec	Increase of 1%
2	60	1 min	
3	95	30 sec	
4	60	15 sec	

Table 2-6. qPCR Programme

Gene (Accession No.)	qPCR Primer	Sequence	Position	Reference
GAPDH (NM_001206359.1) Gene ID: 396823	F	TCAACGACCACTTTGTCAAGC	Exon (1002-1022)	In house
	R	GGTGGTCCAGGGGCTCTTA	Exon (1100-1118)	
MX1 (NM_214061.2) Gene ID: 397128	F	GGTCAGAGAGAAGGAGGC	Exon (1801-1818)	In house
	R	CCGACCTCCTGATGGTAG	Exon-Exon (1935-1918)	
MX2 (AB258432.1) Gene ID: 396893	F	GGAGGAGCTCTTCAACCC	Exon (1767-1784)	In house
	R	GAGGTCCCCATGAAGTAC	Exon (1901-1884)	
CXCL10 (NM_001008691) Gene ID: 494019	F	CCCACATGTTGAGATCATTGC	Exon (239-259)	(Fishbourne, Abrams <i>et al.</i> 2013)
	R	CATCCTTATCAGTAGTGCCG	Exon (406-387)	
PKR (NC_010445.4) Gene ID: 397588	F	GAGAAGGTAGAGCGTGAAG	Exon (1119-1137)	(Loving, Brockmeier <i>et al.</i> 2006)
	R	CCAGCAACCGTAGTAGAG	Exon (1193-1176)	

Table 2-7. Primers used for qPCR analysis. All forward and reverse primers were in different exons. F = Forward, R = Reverse.

2.10 Statistical Analysis

GraphPad Prism Version 8.4.2 (GraphPad Software) was used to for statistical analysis. Two-way ANOVA Tukey's multiple comparison test was applied to data with two independent variables, such as unstimulated or stimulated conditions. For analysis of different confocal microscopy categorical observations Fisher's exact test was applied. For stimulation with different isolates at a single time point Tukey's multiple comparison was applied.

3 BIOINFORMATIC ANALYSIS OF THE MGF360 & MGF505 GENES

3.1 Introduction

Attenuation of ASFV isolates lacking MGF360-10L, 11L, 12L, 13L, 14L and MGF505-1R, 2R, 3R have been shown to stimulate an increased type-I IFN response and a reduced replicative ability in the presence of IFN, relative to virulent parent isolates containing the genes (See 1.2.4) (Afonso, Piccone *et al.* 2004, Golding, Goatley *et al.* 2016, Reis, Abrams *et al.* 2016). This provides some insights regarding the molecular function of the said MGF360 and MGF505 proteins as it demonstrates their potential responsibility for the subversion of innate immunity by inhibiting the type-I IFN response. The specific mechanisms of which however remains unknown.

Motifs and domains are often conserved between various proteins and can indicate similar functions. Thus, by comparing our proteins of interest to databases of known proteins and motifs, computational analysis has the capacity to reveal functional and structural motifs that the MGF360 and MGF505 genes may have in common with previously described proteins. This could thus provide invaluable insights and lead the direction of the investigation. I utilised a variety of computational software, including BLASTp, Clustal Omega, SWISS-MODEL, Jpred and HMMScan to search for such motifs. With each programme offering individual insight to the potential function of the MGFs. For instance,

SWISS-MODEL, uses known proteins structures to act as a template to build putative three-dimensional models, whereas HMMScan uses the PFAM database of protein motifs to search the input sequence for known motifs.

3.2 Results

3.2.1 BLASTp Provided no Proteins of Significant Homology but Pointed Towards the Presence of Ankyrin (ANK) Repeats

BLASTp provided the most basic insights into the MGFs of interest. This software highlights regions of similarity of input amino acid sequences against known proteins allowing the user to make comparisons between the two. This may reveal similarity to regions of proteins with a known function and thus giving insight to function of the input. No proteins of similarity have been reported for the MGFs of interest thus far. However, as the BLASTp database of proteins is under constant expansion it was considered worthwhile to attempt these screens using the non-redundant protein database containing 269435541 protein sequences, up to date at the time of writing. MGF360-10L, 11L, 12L, 13L, 14L and MGF505-1R, 2R, and 3R of Georgia2007/1 amino acid sequences were used as input and searched against non-redundant protein sequences. As ASFV proteins would have a high similarity to our input they would therefore show as the highest scoring hits, risking obscuring non-ASFV proteins. Data from the African Swine Fever Virus Taxid: 10497 was therefore excluded to prevent this.

The hit returned by BLASTp did not reveal any proteins of significant similarity, however the results did reveal regions of interest. Both MGF360-11L and 14L

showed no proteins of significant similarity. Regions of MGF360-10L (60-97aa) and 12L (40-86aa) however each matched to an individual protein ankyrin partial [Anaeromyces robustus] ORX75274.1 (45% identity), and LOW-QUALITY PROTEIN: ankyrin repeat and SAM domain-containing protein 6 [Rhinatrema bivittatum] (44% identity) respectively. Interestingly both contain ANK repeats. MGF360-13L (99-159aa) returned tRNA preQ1(34) S-adenosylmethionine ribosyltransferase-isomerase QueA [Campylobacter lari] (43% identity) (See Supplementary Figure - Table 11-1).

Residues 142-193 of MGF505-1R aligned to a group of protein of unknown function, hypothetical protein [Helicobacter pylori] with up to 33% identity. MGF505-2R showed a single hit at residues 213-298 with AAA family ATPase [Ferruginibacter sp. BO-59] with 30% identity. MGF505-3R however showed more varied results, with 6 leucine rich repeat serine/threonine protein kinase proteins and 10 hypothetical or proteins of an unknown function identified as containing regions of significant similarity. The hit with the highest identify (48%) was leucine-rich repeat serine/threonine-protein kinase 1 [Anoplophora glabripennis] which aligned to residues 40-192 of MGF505-3R (See Supplementary Figure - Table 11-1). Although this specific region of this exact protein is yet to be annotated, its human ortholog Leucine-rich repeat serine/threonine-protein kinase 1 contains a number of ankyrin repeat motifs.

3.2.2 Putative Structural Analysis and Motif Homology Indicates the Presence of Ankyrin Repeats

Utilizing SWISS-MODEL ProMod3 modelling engine, an automated structural homology modelling server, I computationally investigated putative three-dimensional tertiary structures of the proteins encoded by the MGF360 and MGF505 target genes. This provided valuable insight into potential molecular function. The engine is capable of generating a predicted tertiary structure by aligning target gene amino acid sequences to that of a database (SWISS-MODEL Template Library, an aggregate of the Protein Data Bank) of proteins with a known structure, known as 'templates'. Appropriate templates are then selected based upon a scoring metric "Global Model Quality Estimation" (GMQE). GMQE is used to indicate expected model accuracy on a scale of 0-1. Finally, a model of the 3D structure of the input is built upon the structure of the template. As this is based on similarities between the input and template, the resulting model may only cover a region of similarity of the input, rather than the entire protein. The quality of said model is estimated via QMEAN Z-score, inferring statistical significance and models "nativeness" relative to proteins of similar size. QMEAN takes four different scores into account, comparison of the interaction between C β atoms, all atoms, solvation, and torsion angle, with scores lower than -4.0 considered low quality (Waterhouse, Bertoni *et al.* 2018).

Using this engine, I analysed MGF360-10L, 11L, 12L, 13L, 14L, and MG505-1R, 2R, 3R sequences from Georgia2007/1. Only MGF360-11L, 12L and 14L sequences returned a model with a QMEAN Z-score score considered to indicate a high quality and thus accurate model. Both MGF360-11L and 14L matched to Poly-ADP-ribosyltransferase Tankyrase-1. However, despite showing low confidence with the models created for the remaining proteins, each model generated from the automatically selected templates showed a constant theme of similarity to ANK repeats. Furthermore, the previously mentioned notable template, Tankyrase-1 contains 5 clusters of ANK repeats (Eisemann, McCauley *et al.* 2016). The model generated for both was built from templates 3utm.1.A (QMEAN -1.73, GMQE 0.10) Tankyrase-1 amino acid region 129-300, or 3utm.1.B (QMEAN -2.91, GMQE 0.09) Tankyrase-1 amino acid region 182-302 respectively. Notably this region codes for 4 ANK repeats. The modelled region aligned to MGF360-11L region 187-312aa, and MGF360-14L region 208-309aa. Additionally, MGF360-14L resulted in a model being built from a genetically engineered ANK repeat protein (DARPin) variant DDD_D12_06_D12_06_D12 (5leb.1.A, QMEAN -3.70, GMQE 0.15) amino acid region 273-405 that aligned to residues 182-308 of 14L (Figure 3-1). Additionally, MGF360-12L generated a model based on Ankyrin Repeat SOCS9 in complex with EloB and EloC (3zgj.1.A, QMEAN -0.04, GMQE 0.02) for residues 60-111. However, despite the QMEAN score for the models built were considered high quality, the GMQE values were low indicating low accuracy of the models.

Non-significant models however mapped to much larger regions of both the MGF360 and MGF505 clusters. For MGF360-10L, 11L, 12L, 13L, the largest

mapped region was Nav1.2 – AnkB chimera (4rly.1.A, QMEAN= -5.56 to -4.40). For the MGF360-14L protein, 2-5A-dependent ribonuclease (4g8l.1.A, QMEAN= -4.40) was the template that covered the largest region. Both templates covered regions of approximately 55-310 amino acids across the MGF360 cluster. The modelled region maps to a section of up to 9 ANK repeats. All MGF360 proteins also had models built using DARPin templates. DARPins are a genetically engineered antibody mimetic consisting of repetitive ANK repeats (Plückthun 2015). Templates included R12 (6mol.1.A), DDD_D12_06_D12_06_D12 (5leb.1.A), DD_D12_13_D12 (5le7.2.A), and DARPin1238_E11 (6fp8.1.B). Similarly, the MGF505 proteins matched to templates containing numerous ANK repeats. Notably, like the MGF360 cluster, a common template used for models were numerous variations of DARPins. DARPin proteins variants were the basis for models approximately mapping to MGF505-1R residues 55-500, MGF505-2R residues 55-375, and MGF 505-3R residues 55-275.

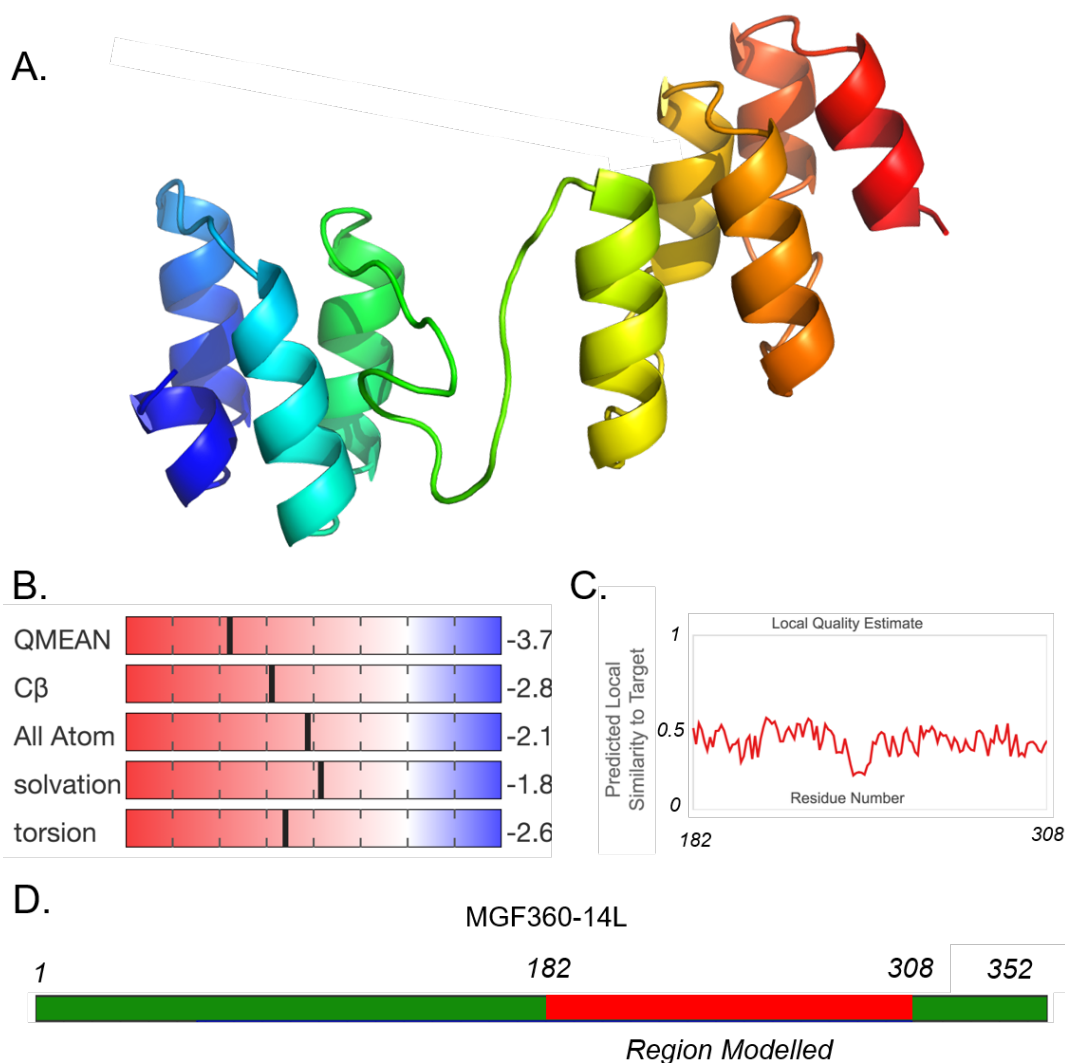


Figure 3-1. MGF360-14L SWISS-MODEL built against DARPin variant DDD_D12_06_D12_06_D12 (5leb.1.A) **A.** Cartoon representing resulting model predicting the tertiary structure of MGF360-14L. **B.** QMEAN score, and breakdown of the four constituent components that make up the total score. White shading represents a score of 0. Scores are noted on the right-hand side. Scores above -4.0 are considered of a high quality. **C.** Chart showing the similarity score of residues of the model (x-axis) vs. MGF360-14L (y-axis). Score of 0.6 and above is considered of high quality. **D.** Crude positional alignment of model built based on residues 237-405 of 5leb.1.A (red bar), corresponding to residues 187-312 of MGF360-14L.

In order to further investigate the predicted presence of ANK repeats, HMMER version 3.3 HMMscan software was used to analyse the MGF proteins against known motifs within the PFAM HMM library. As expected, the results for each protein showed the presence of a motif consistent with their corresponding MGF360 or 505 cluster (ASFV_360 (MGF360) PF01671.16, or DUF249 (MGF505) PF03158.13). Similarly to SWISS-MODEL, MGF360-10L, 11L, 12L, MGF505-1R, 2R, 3R, 4R and 5R also returned numerous ANK repeat motifs (Table 3-1). Interestingly MGF360-13L and 14L did not contain predicted ANK repeats. Furthermore, the predicted regions containing the ANK motifs also roughly corresponded to that predicted by SWISS-MODEL. The results showed 3-11 ANK repeats across the length of the proteins starting between residues 37-63. Although most individual ANK repeat motif hits returned by the software were deemed significant, a number of the motifs, despite falling within the defined E-Value threshold, bit scores (defined as “log of ratio of the sequences probability according to homology hypothesis to the null model probability”) fell below the databases threshold, and thus were classified as insignificant hits.

Taken together the results from SWISS-MODEL and HMMScan indicated the presence of numerous ANK repeats in all but two (MGF360-13L, and 14L) of the MGF proteins analysed. The numbers of ANK repeats varied between 0 and 5 for proteins of the MGF360 cluster and 6 to 11 for those of MGF505. This provides interesting insight into the potential functional role of the proteins. ANK repeats are well described as facilitating protein-protein interactions (Mosavi, Cammett *et al.* 2004). Therefore, this computational data suggesting the presence of ANK

repeats within the MGFs analysed reveals that they may be involved in a similar role. This potentially indicates that inhibition of the IFN response by MGF360 and MGF505 proteins may be caused by direct targeting of specific components of the pathway.

MGF of Interest	Approximate No. of Predicted ANK Repeats	Predicted Residues
MGF360-10L	5	57-87, 158-236, 239-266
MGF360-11L	3	63-87, 210-235, 237-267
MGF360-12L	4	39-87, 180-232
MGF360-13L	0	
MGF360-14L	0	
MGF505-1R	6	57-87, 133-203, 237-341
MGF505-2R	7	58-83, 101-188, 228-310
MGF505-3R	4	38-86, 168-275
MGF505-4R	11	37-90, 100-182, 235-319, 327-442, 457-501
MGF505-5R	9	55-115, 135-230, 231-316

Table 3-1. HmmScan predicted presence of ANK repeats within MGFs Predicted residues represents the contiguous presence of the motif within the protein.

3.2.3 Both MGF360 & MGF505 Cluster Proteins have a Putative VHL/SOCS-Box-Like Motif

ANK repeats are widely abundant in eukaryotes, but less so in prokaryotes, and archaea (Mosavi, Cammett *et al.* 2004, Jernigan and Bordenstein 2014). Their presence within viruses is generally limited to poxviruses, specifically in those belonging to the chordopoxvirus order where ANK containing proteins make up to 15% of the proteome (Mosavi, Cammett *et al.* 2004, Mercer, Fleming *et al.* 2005, Sonnberg, Seet *et al.* 2008, Herbert, Squire *et al.* 2015). Similarly to the MGFs of ASFV, the poxvirus ANK containing proteins are typically found close to the terminal ends of the viral genome and commonly play a role in virulence and host determination (Herbert, Squire *et al.* 2015). Poxvirus proteins containing ANK repeats are often large proteins of over 400 residues and have a consistent domain architecture. The N-terminus is formed of 4-10 ANK repeats, often associated with a short motif at the C-terminus, that shares sequence and functional similarity to the eukaryotic F-Box, known as a PRANC motif for CRL recruitment (See 1.1.7.4). Due to the presence of ANK motifs in the MGFs of interest and the additional similarities with the PRANC proteins I proposed that they may be functioning in a similar manner. I therefore began the process of analysing MGF amino acid sequences for the presence of potential F-Box PRANC-like motifs.

Using PFAM motif finder and ClustalOmega to align MGF sequences to the cellular F-Box motif consensus sequence (F-X-L-L-X-L-P-X(3)-L-X(2)-I-L-X(2)-L-X(8)-R-X-V-S-X(3)-R) (Kipreos and Pagano 2000), or the viral PRANC motif

consensus sequence (L-P-X-E-[IVL]-[VL]-X(2)-[IV]-L-X(2)-[VL]-X(4)-L) (Mercer, Fleming *et al.* 2005, Sonnberg, Seet *et al.* 2008), I was unable to identify a poxvirus-like F-Box motif within the MGF proteins of interest (Data not shown).

Although PRANC motifs use ANK repeats as substrate binding partners, cellular F-Box proteins have not been observed to do so. Nevertheless, it is of note that CRL substrate receptor binding proteins VHL and SOCS proteins contain a F-Box-like motif known as VHL-Box or SOCS-Box respectively, and do utilize ANK repeats as substrate binding partners (See 1.1.7.4 and Figure 1-3). (Kamura, Maenaka *et al.* 2004, Mahrour, Redwine *et al.* 2008, Muniz, Guo *et al.* 2013). Although highly similar the VHL-Box and SOCS-Box can be further divided into constituent components, a BC-Box, and a Cul binding region (Cul2-Box is archetypal of a VHL-Box, whereas a Cul5-Box is of a SOCS-Box) (See 1.1.7.4 and Figure 1-3). I therefore investigated the presence of a VHL-Box or SOCS-Box motif within the MGFs. By using ClustalOmega to align protein sequences from the MGF360 and MGF505 proteins against numerous known VHL-Box or SOCS-Box motifs (Figure 3-2). Here I identified a novel N-terminus VHL-Box or SOCS-Box-like motif in both MGF clusters, although which of the two motifs specifically present was not apparent. The canonical BC-Box [TSP]-X-X-X-[CSA]-X-X-X-[Φ] (where Φ indicates hydrophobic residues) was clearly present within the MGF505 cluster, and partially so in the MGF360 cluster (Figure 3-2). However, this alignment revealed the presence of a Cul2-Box or Cul5-Box motif, although specifically which is present is unclear (Figure 3-2). Interestingly the intact BC-Box found in MGF505 proteins, when aligned to the BC-Box present in the VHL-

Box and SOCS-Box containing proteins showed a higher sequence similarity to that of the SOCS-Box (Figure 3-3A). Likewise, phylogenetic analysis of these domains indicated the same (Figure 3-3B).

APBP2	-----PE	TYNTAISAVVDNYIR-SR-----	RDIRS	LPEN	IQFD	YVY----	35		
LRR1	-----PL	TLLSEARTILHNRIP-YG-----	SHIL	EFHC	QDID	-----	33		
FEM1B	-----KMS	LKCLAAARVRANDIN-YQ-----	DQIP	R-TLE	EFVG	-----	32		
FEM1A	-----VT	LQCLAAARALDKNKIP-YK-----	GFIPE	DLE	AFIE	-----	31		
FEM1C	-----HT	LQCLAAARVIVNHRIY-YK-----	GHIEP	KLE	TFVS	-----	32		
MUF1	-----PP	ALFELCGRAVSAHMGV--LE-----	SGV	WALP	GPILO	SIL-PL-----	37		
KLHDC2	-----PK	SLVRLSLEAVICFKEM--LA-----	NSWNC	LKH	ILH-SVN	-----	35		
LRR28	-----VL	PLQELAMRGLYHTYHS-----	LLKDLN	--FL-SP-IS	LPR-SLL-EL	ELH-----	40		
MGF505-4R	-----MF	SLQDICRKHL--FQLPDAFDEYILQALG--	LYWEK	HGSLQ	RIRKDAV	FVQRN--	50		
MGF505-2R	-----MF	SLQDLCKRHL--FILPDVFGEHVLQRLG--	LYWRC	HGSLQ	RIGDDHIL	RRD--	50		
MGF505-5R	-----MF	SLQEICRKNI--YFLPDWLGEHVIQRLG--	LYWEK	HGSLQ	RIGDNYVLI	QD--	50		
MGF505-1R	-----MF	SLQNLCKRTLPRNKLPEFFDEYILQLLG--	LYWEN	HGTIQ	RAGNNCVLI	Q--	50		
MGF505-3R	-----MSS	SLQELCRKKLPDCILPEFFDYVLQLLG--	LHWQD	HGSLQ	RIKKNQILV	----	50		
LLRC42	-----AK	SLFSLVLGFTSDNVHD-ID-----	SL-IG	FEE	QIA-EK	LF-----	34		
EloA	-----MM	TLHQQCIRVLKNNIDS-IF-----	EV-GG	VPY	S	LEPVL-----	34		
MGF360_14L	-----ML	SLQTLAKKVACNYLS-SDYDYLQRF--	LWWD	L	GPIHL	CNNCKQV	FYSYK--	50	
MGF360_13L	-----MS	LPLSLQTLVKKTIASQCLS-IDEHCILKYCG--	LWWHD	APLKL	CMDRGRI	Q--	50		
MGF360_10L	-----MF	PSLQSFQAKKVLARQVS-IEYHVILERC--	LWYK	APISL	DKHMLIK	L	P--	50	
MGF360_11L	-----ML	PSLQSLTKKVLQGCIP-VDQYHVLKCCG--	LWWHN	GPIML	HIRRNKL	FIR--	50		
MGF360_12L	-----ML	PSLQSLTKKVLQGCVP-TNQHYLLKCYD--	LWWHD	APITF	DHNLRLI	KSA--	50		
LRR14	-----MH	TLVFLSTROVLQCQPA-----	ACQAL	PL	LPR	E	F-PLLF-----	35	
SOCS3	-VLSRPLSSNVATL	QHLCKRTVNGHLS-----	YEKVT	--QLPG	PIR	EF	LQYDAP	48	
VHL	-----VY	TLKERCLQVRSVLKVP--ENY-R-----	RL--D	IVR	S	Y-ED	IE-----	35	
ZYG11BL	-----PE	SMLALCTDFCLRNLDG-TLGY-LLDKETLRLHPDI--	FLPS	E	C	DR	LV-----	46	
KLHDC3	-----SP	SKTLCKLAVIQYNLD-----	QS--C	LPH	D	R	WE	LN-----	31
ASB1	-----PR	TLLCLCRVAVRRALGK--HRL-----	HLIPSL	--PLPD	P	IK	K	FLL-----	38
ASB4	HSLFTVCCNSPRT	LMHLSRCAIRRTLHN--RCH-----	RAIPIL	--SLPL	SLK	KYLL	LEPEG	53	
ASB6	-----PP	PLKHLRCRAIRLYLQP---W-P---	VDVKVKAL	--PLPD	RLK	WYLLS	----	40	
ASB3	-----VP	SLTHLCRLERSSLSK--ERL-R---	SDSY	TSQ	--PLPR	S	LH-NYLL-----	41	
SOCS2	-----AP	SLQHLCLRTINKCTG-----	AIWGL	--PLPT	RLK	D	YLE-----	33	
WSB1	-----VP	SLQHLCRMSIRRVMT-----	QEVQEL	--PIPS	KLL	E	FLS-----	35	
SOCS4	LLSTPLIRTFPFS	LQHICRTVICNCTTY-----	DGIDAL	--PIPS	SMK	LYL	KEYHYK	50	
SOCS5	LLTISLNRTPFFS	LQYICRAVICRCTTY-----	DGIDGL	--PLPS	MLQ	D	FLKEYHYK	50	
SOCS6	-----VR	SLOYLCRFVIRQYTRI-----	DLIQKL	--PLPN	KMK	D	YLQ-----	35	
SOCS7	-----VK	SLQHLCRFRIRQLVRI-----	DHIPDL	--PLPK	PLI	S	YIR-----	35	

Figure 3-2. Alignment MGF360 and MGF505 proteins with various VHL-Box and

SOCS-Box proteins. 50 N-terminus amino acids of MGF360 and MGF505 cluster

are aligned with the VHL-Box (cyan) or SOCS-Box (red) motifs of numerous proteins. Critical residues of the BC-Box motif are in yellow, highlighting the exceptional homology with the ASFV proteins. Cul2-Box motif residues are highlighted in green, and Cul5-Box in magenta. MGF residues that share similarity with critical residues of either Cul2 or Cul5 motifs are shaded in grey.

A.

MGF505-4R	-----MFSLQDLCRKHL--FQLPDAFDEYILQALGLYWEKHGSLQRIKDAVVFVQRN	50
MGF505-2R	-----MFSLQDLCRKHL--FILPDVFGEHVLQRLGLYWCHGSLQRIQDDHILIRRD	50
MGF505-5R	-----MFSLQELCRKNI--YFLPDWLGEHVIQRLGLYWEKHGSLQRIQDNYVLIQQD	50
MGF505-1R	-----MFSLQNLCKRTLPNRKLPEFFDEYILQLLGLYWENHGTIQRAGNNCVLIQ--	50
MGF505-3R	-----MSSSLQELCRKLPDCILPEFFDDYVLQLLGLHWQDHGSLQRIEKNQILV---	50
KLHDC2	-----PKSLVRLSLEAVICFK-----	16
ZYG11BL	-----PESLMALCTDFCLRNL-----	16
LLRC42	-----AKSLFSLVLGFISDNV-----	16
VHL	-----VYTLKERCLQVVRSLV-----	16
ASB1	-----PRTLCLCRVAVRRALGKHLH-----	22
ASB4	HSLFTVCCNSPRTLMLHSRCAIRRTLHNRCHRAI-----	34
KLHDC3	-----SPSLKTLCKLAVIQYN-----	16
ASB6	-----PPPLKHLCRVAIRLYLQWPVPD-----	22
SOCS3	VLSRPL-SSNVATLQHLCKRTVNGHLDYS-----	28
SOCS6	-----VRSLOYLCREVIRQYTRI-----	18
SOCS7	-----VKSLQHLCRFRIRQLVRI-----	18
ASB3	-----VPSLTHLCRLEIRSSSLKSERLRSD-----	24
WSB1	-----VPSLQHLCRMSIRRVMP-----	18
SOCS2	-----APSLQHLCRILTINKCT-----	16
SOCS4	LLSTPLIRTFPFSLOHICRTVICNCTTY-----	28
SOCS5	LLTISLNRTFPFSLOYICRAVICRCTTY-----	28
LRRC14	-----MHTLVFLSTRQVLQCC-----	16
EloA	-----MMTLHQQCIRVLKNNID-----	17
APPBP2	-----PETLYNTAISAVVDNY-----	16
MUF1	-----PPALFELCGRAVSAHMGVLE-----	20
LRR1	-----PLTLLESSARTILHNR-----	16
LRRC28	-----VLPLQELAMRGLYHTY-----	16
FEM1C	-----HTTLQCLAARVIVNHR-----	16
FEM1B	-----KMSLKCLAARAVRAND-----	16
FEM1A	-----VTTLQCLAARALDKNK-----	15
BC-Box	-----tL---c---φ-----	
	s a	
	p s	

B.

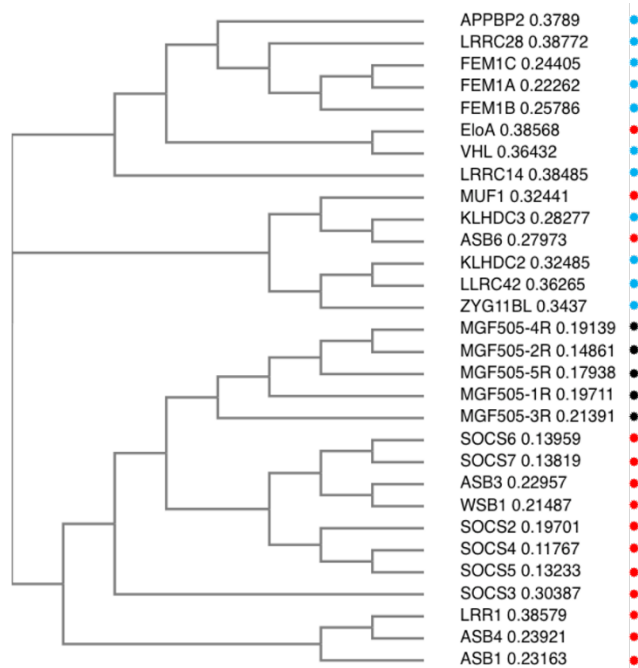


*Figure 3-3 Comparison of MGF505 BC-Box motif to that of known VHL-Box and SOCS-Box proteins. **A.** Alignment of MGF505 proteins with the BC-Box of VHL-Box (cyan) and SOC-Box (red) containing proteins. Crucial residues of the motif are highlighted in yellow. Additional conserved residues are in bold and shaded dark grey, and those with similarity in light grey. BC-Box consensus sequence is shown at bottom of alignments. **B.** Phylogenetic tree showing the similarity between the BC-Box and Cul-Box of MGF505 (black star) to that of SOCS proteins (red star), rather than VHL (cyan star).*

The presence of the BC-Box adjacent to Cul2-Box is a hallmark of a VHL-Box, as opposed to the SOCS-Box which utilizes the BC-Box and Cul5-Box. As the presence of either Cul binding motif was unclear, each cluster was analysed for each a Cul2-Box [Φ -P-X-X- Φ -X-X- Φ], or a Cul5-Box [Φ -X-X-LP Φ -X-X- Φ -X-X-X- Φ] alone (Figure 3-4AB, Figure 3-5AB). MGF360 proteins contained a partial or degenerate Cul2-Box motif, while the complete motif was present in only MGF360-12L (Figure 3-4A). A partial Cul5-Box motif was also found, again with MGF360-12L showing the most complete motif (Figure 3-4B). The presence of a fully formed Cul2-Box however was visible in the MGF505 proteins (Figure 3-5A). Interestingly however, it would appear the Cul5-Box motif may also be present although the sequences match the motif less clearly than the Cul2-Box (Figure 3-5B).

Taken together this data indicates that the motif found in the MGF360 and MGF505 proteins is a novel variant of either the VHL-Box or SOCS-Box. However, the vagueness of the Cul bind region means it is difficult to explicitly say which of the two CRL recruitment motifs are present, or whether a hybrid of both. Functional confirmation is required to determine the specific Cul binding partners. From here onwards, until specifically determined the indeterminate Cul binding region of the MGFs will be referred to as a Cul2/5-Box.

A.

ZYG11BL	-----HPDIFLPSEICDRV-----	15
KLHDC2	-----NSWNCLEPKHLLHSVN-----	15
LLRC42	-----DSLIGFPEQIAEKLF-----	15
LRRC14	-----QALPLLPRELFPLLF-----	15
LRRC28	-----LSPISLPRSLLELLH-----	15
FEM1B	-----NYQDQIPRTLEEFVG-----	15
FEM1A	-----PYKGFIPEDLEAFIE-----	15
FEM1C	-----YYKGHIPEKLETFVS-----	15
VHL	-----YRRLDIVRSLEYEDLE-----	15
LRR1	-----YGSHIIPFHLCDQLD-----	15
KLHDC3	-----LDQSCLEPHDIRWELN-----	15
APPBP2	-----RDIRSLPENIQFDVY-----	15
MGF360-14L	---MLSLQTLAKKVACNYLSSDYDYTLQRFGLWWDLGPIHLCNNCKQVFSYK	50
MGF360-13L	MSLPLSLQTLVKKTIASQCLSIDEHCILKYCGLWWHDAPLKL CMDRGRIQ---	50
MGF360-10L	--MFPSLQSFQKKVLARQQVSI EYHVILERCGLWWYKAPISLDCKHMLIKLP-	50
MGF360-11L	--MLPSLQSLTKKVLAGQCIPVDQYHVLKCCGLWWHNGPIMLHIRRNLKLFIR-	50
MGF360-12L	--MLPSLQSLTKKVLAGQCVPTNQHYLLKCYDLWWHDAPITFDHNLRLIKSA-	50
Cul2 Box	-----φP--φ--φ-----	

B.

MUF1	-----SGVWALPGP--ILQSLPL-----	17
SOCS3	-----EKVTQLPGPIREFLDQYDAP-	20
WSB1	-----QEVQELPIPSKLEFLS-----	17
SOCS7	-----DHIPDLPLPKPLISYIR-----	17
ASB3	-----SYISQLPLPRSLHNYIL-----	17
ASB4	-----IPLLSLPLSLKKYLLLEPEG-	20
ASB1	-----LIPSLPLPDPIKKFLL-----	16
ASB6	-----VKVKALPLPDRLKWYLLS-----	18
SOCS6h	-----DLIQKLPLPNKMKDYLO-----	17
SOCS2	-----GAIWGLPLPTRLDYLE-----	17
SOCS4	-----DGIDALPIPSMKLYLKEYHYK-	22
SOCS5	-----DGIDGLPLPSMLQDFLKEYHYK-	22
EloA	-----SIFEVGGVPYSVLEPVL-----	17
MGF360-14L	---MLSLQTLAKKVACNYLSSDYDYTLQRFGLWWDLGPIHLCNNCKQVFSYK	50
MGF360-13L	MSLPLSLQTLVKKTIASQCLSIDEHCILKYCGLWWHDAPLKL CMDRGRIQ---	50
MGF360-10L	--MFPSLQSFQKKVLARQQVSI EYHVILERCGLWWYKAPISLDCKHMLIKLP-	50
MGF360-11L	--MLPSLQSLTKKVLAGQCIPVDQYHVLKCCGLWWHNGPIMLHIRRNLKLFIR-	50
MGF360-12L	--MLPSLQSLTKKVLAGQCVPTNQHYLLKCYDLWWHDAPITFDHNLRLIKSA-	50
Cul5-Box	-----φ--LPφP--φ--φ-----	

Figure 3-4. Alignment of MGF360 proteins against sequences of known Cul-Box motifs Alignment against proteins containing **A. VHL-Box** proteins (cyan) **Cul2-Box** (Green) or **B. SOCS-Box** proteins (red) **Cul5-Box** (Magenta) motif. **Cul2-Box** and **Cul5-Box** consensus sequences are shown at bottom of alignments.

A.

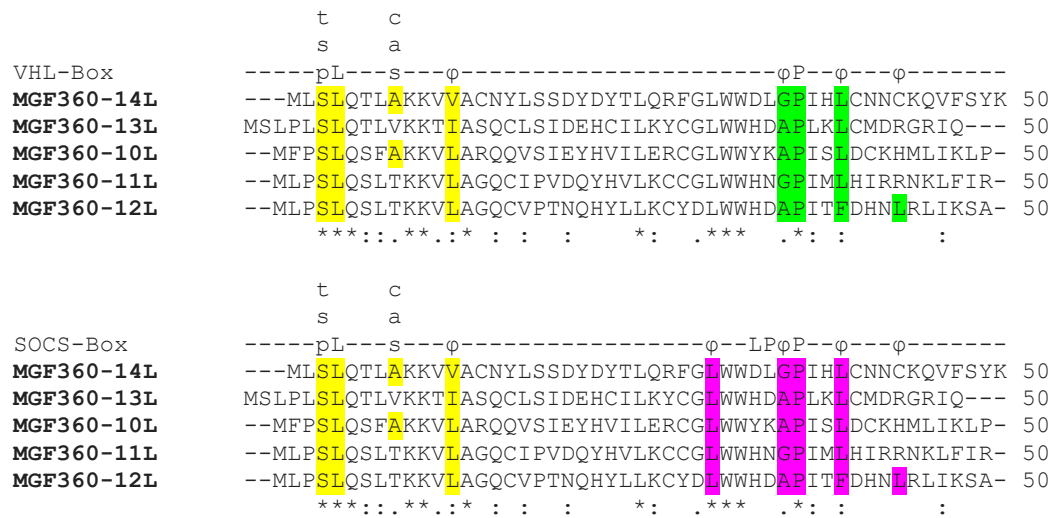
FEM1B	-----NYQDQIFRTIEEFVG-----	15
FEM1A	-----PYKGFIPEDIEAFIE-----	15
FEM1C	-----YYKGHIEKLETFVS-----	15
VHL	-----YRRLDIVRSIYEDIE-----	15
LRR1	-----YGSHIIEFHICQDLD-----	15
APBP2	-----RDIRSLPENIQFDVY-----	15
MGF505-2R	-MFSLQDLCKHL--FILDVVEGEHVLQRLGLYWCHGSLQIRIGDDHILIRRD	50
MGF505-5R	-MFSLQELCKRNI--YFLPDWVGHEVILQRLGLYWEKHGSLQIRIGDNYVLIQQD	50
MGF505-4R	-MFSLQDLCKHL--FQLPDADFDEYILQALGLYWEKHGSLQIRIKDAVQVQRN	50
MGF505-1R	-MFSLQNLCKRTLPNRKLPEFFDEYILQLLGLYWENHGTIQRAGNNCVLIQ--	50
MGF505-3R	MSSSLQELCRKKLPDCILPEFFDDYVLQLLGLHWQDHGSLQRIEKNQILV---	50
KLHDC3	-----LDQSCLEPHDIRWEIN-----	15
ZYG11BL	-----HPDIFLESEICDRIV-----	15
KLHDC2	-----NSWNCLEPKHLHSHN-----	15
LLRC42	-----DSLIGFPEQLAEKLF-----	15
LRRC1	-----QALPLLEREIFPLIF-----	15
LRRC28	-----LSPISLEERSILELH-----	15
Cul2-Box	-----φP--φ--φ-----	

B.

ElcA	-----SIEVGGVPSYVLEPVL-----	17
MGF505-4R	-MFSLQDLCKHL--FQLPDADFDEYILQALGLYWEKHGSLQIRIKDAVQVQRN	50
MGF505-2R	-MFSLQDLCKHL--FILDVVEGEHVLQRLGLYWCHGSLQIRIGDDHILIRRD	50
MGF505-5R	-MFSLQELCKRNI--YFLPDWVGHEVILQRLGLYWEKHGSLQIRIGDNYVLIQQD	50
MGF505-1R	-MFSLQNLCKRTLPNRKLPEFFDEYILQLLGLYWENHGTIQRAGNNCVLIQ--	50
MGF505-3R	MSSSLQELCRKKLPDCILPEFFDDYVLQLLGLHWQDHGSLQRIEKNQILV---	50
MUF1	-----SGVWALPGP--ILQSTLPL-----	17
SOCS3	-----EKVTQLPGPIREFLDQYDAP-----	20
WSB1	-----QEVQELPIPSKLLFLS-----	17
SOCS7	-----DHIPDLPLPKPLISYIR-----	17
ASB3	-----SYISQLPLPSLHNYLL-----	17
ASB4	-----PLLSPLSLKKYLLLEPEG-----	19
ASB1	-----LIPSLPLPDPIKKFLL-----	16
ASB6	-----VKVKALPLPDRLKWLIS-----	18
SOCS6h	-----DLIQKLPLPNKMKDYLLQ-----	17
SOCS2	-----GAIWGLPLPTRIKDYLL-----	17
SOCS4	-----DGTIDALPIPSMKLYLKEYHYK-----	22
SOCS5	-----DGTIDGLPLPSMLQDFLKEYHYK-----	22
Cul5-Box	-----φ--LPφP--φ--φ-----	

Figure 3-5 Alignment of MGF505 proteins against sequences of known Cul-Box motifs Alignment against proteins containing **A.** VHL-Box proteins (cyan) Cul2-Box (Green) or **B.** SOCS-Box proteins (red) Cul5-Box (Magenta) motif. Cul2-Box and Cul5-Box consensus sequences are shown at bottom of alignments.

A.



B.

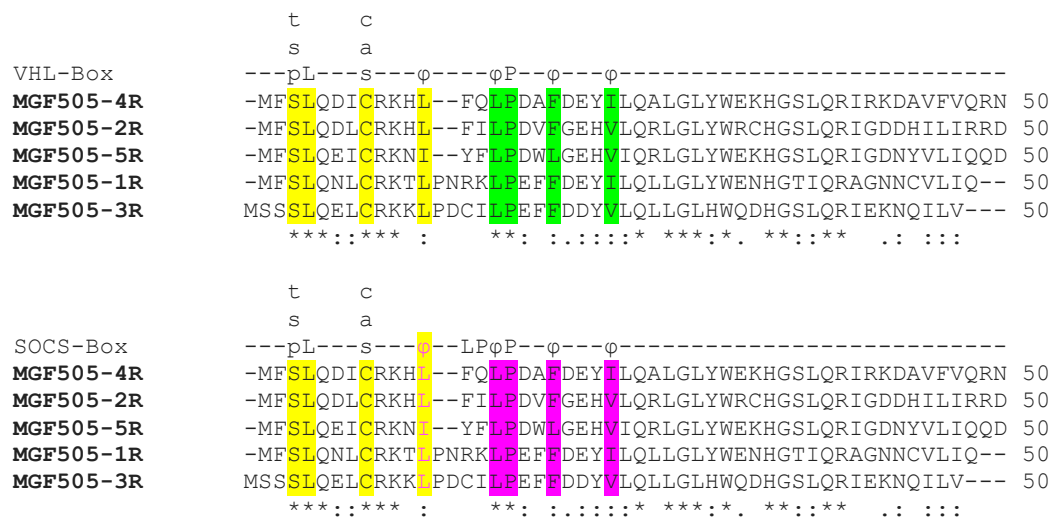
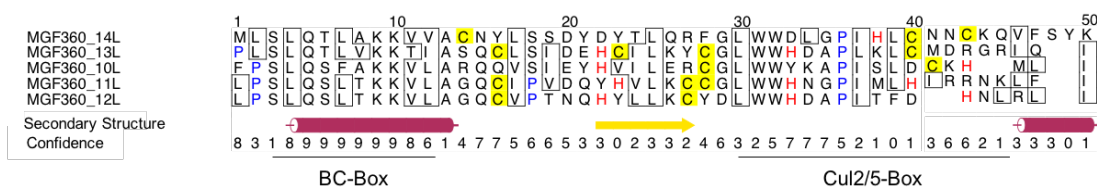


Figure 3-6. Alignment of MGF360 and MGF505 proteins highlighting the VHL-Box and SOCS-Box consensus sequence. Highlight of VHL/SOCS-Box consensus motifs in the N-terminus residues of the **A.** MGF360 proteins and **B.** MGF505 proteins. VHL-Box or SOCS-Box consensus sequences are shown at top of alignments. Overlapping residues are highlighted in yellow with magenta text. Residue homology is shown beneath. * indicates similarity, : indicates highly conserved, . indicates conserved.

3.2.4 Predicted Structure of the BC-Box & Cul2/5-Box of the MGFs Show Similarity to that of Cellular Counterparts

Using Jpred v4 software MGF505 proteins were predicted to have two rather than three helices falling at the predicted VHL/SOCS-box (Figure 3-7B). However, an α -helix at the putative BC-Box, and a second α -helix adjacent to what resembles the LP residues of the motif and covering the remainder of the putative Cul2/5-Box match the location of the two crucial helices that form the key hydrophobic interactions required for interactions to form the EloB/C complex in SOCS proteins. The MGF360 proteins displayed a similar architecture at the putative BC-Box and Cul2-Box (Figure 3-7A).

A.



B.

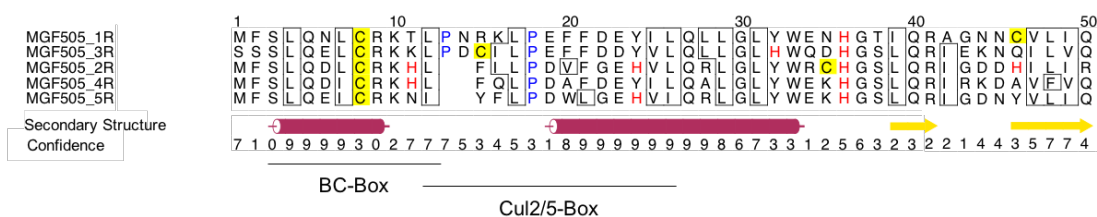


Figure 3-7 . Jpred analysis showing the predicted secondary structure of MGF360 and MGF505 proteins Analysis was performed on the first 50 residues of **A.** MGF360 and **B.** MGF505 proteins matching region of BC-Box and Cul2/5-Box. Burgundy tubes indicate helices; yellow arrows indicate sheets. Confidence (0-10) gives estimate of confidence for the prediction, with higher values showing higher

confidence. Hydrophobic residues are highlighted in boxes, cysteines in yellow, proline in blue, and His in red.

3.2.5 MGF360-11L, 12L & 14L have a Predicted TRAF6 Binding Motif

I discovered the presence of the non-canonical TRAF6 binding motif [RxExxAr/Ac] where Ar is an aromatic, and Ac is an acidic residue, in both MGF360-11L and 12L at residues 154-159, and 152-157 respectively (Figure 3-8AB). This motif differs from the canonical TRAF6 binding motif where the first glycine is replaced by a proline residue. The non-canonical motif found in MGF360-11L, and 12L coincides with the TRAF6 binding motif of poxvirus A52 (Stack, Hurst *et al.* 2013). Additionally, MGF360-14L contains the canonical motif [PxExxAr/Ac] at residues 325-330 (Figure 3-8C).

A.

MGF360-11L

MLPSLQSLTKKVLAGQCIPVDQYHVLKCCGLWWHNGPIMLHIRRNKLFIRSTCFSQGIE
LNIGLMKAVKENNHDLIKLFTEWGADINYGMICALTENTRDLCKELGAKEYLEREYILK
IFFDTRDKTSSNIIFCHEVFSNNPNLRIIDNLDL**RGEIMW**ELRGLMEITFMLDHDDSF
STVLTKYWYAIADVLDKDAIRYFYQKYPRLHRWRLMCALFYNNVFDLHELVEIERVRM
DIDEMMHIACIQDYSYSAIYYCFIMGANINQAMLVSIQNYNLGNLFFCIDLGADAFEEG
KALAEQKENYLIHAHALSLKHYNPVISLLSIVTDPEKINCMLKNYHSINMGIFLDYEQR

B.

MGF360-12L

MLPSLQSLTKKVLAGQCVPTNQHYLLKCYDLWWHDAPITFDHNLRLIKSAGIKEGLNLN
TALVKAVRENNYNLIKLF~~AEW~~GADINYGLVSVNTEHTWDLCRELGAKETLNEEEILQIF
IDLKFHKTSSNIILCHEVFSNNPILQKVNNIKM**RIEIFW**ELRELIVKTDLLNNEFSLST
LLKYYWYAI~~AI~~RYNLKEAIQYFYQKYTHLNTWR~~LT~~CALCFNNVFDLHEAYEKDKIHMDI
EEMRIACIKDHNLS~~T~~MYCYVLGANINQAMLSSIQYYNIENMFFCIDLGADVFEEGTT
ALGEGYELIKNILSLKIYSPATTPPKSTDPEIIDHALKNYVSKNMMIFLTYDLR

C.

MGF360-14L

MLSLQTLAKKVACNYLSSDYDYTLQRFGLWWDLGPIHLCNNCKQVFSYKHLQCFSEDD
LCLEAALVKAVKSDNLELIRLFVDWGANPEYGLIRVPAVYLKRLCAELGGLTPVSEPR
LEILKEVARLKSCAGVLLGYDMFCHNPLLETVTRTTLDTVTYTCSNIPLTGDTAHHLLT
KFWFALALRHNF~~TK~~AIHYFYKRHKNHLYWRVACSLYFNNIFDIHELCREKEICISPNLM
MKFAC~~L~~REKNYAAIYYCHRLGASLDYGMNLSIYNNNTLNMFFCIDLGAAFDRAQLIAH
KAYMYNLSNIFLVKQLFSRDVTLVLDVTE**PQEITYD**MLKTYTSKNLKRAEEYLTAHPEII
VID

*Figure 3-8. TRAF6 binding motifs in MGF360-11L, 12L and 14L. Amino acid sequences of **A.** MGF360-11L, **B.** 12L and **C.** 14L highlighting non-canonical TRAF6 binding motif [RxE~~xx~~Ar/Ac] in cyan, and canonical TRAF6 binding motif [PxE~~xx~~Ar/Ac] in magenta.*

3.2.6 MGF505-2R has a Predicted IRF3 Binding Motif

ScanProsite motif finder revealed the presence of an IRF3 binding motif pLxIS (where p is a hydrophilic residue) in MGF505-2R at residues 358-362 (Figure 3-9). This motif is found in key adaptor proteins including MAVS, STING, and TRIF for the recruitment of the transcription factor IRF3 (Liu, Cai *et al.* 2015).

MGF505-2R

MFSLQDLCKRHLFILPDVFGHEVLQRLGLYWRCGSLQRIQDDHILIRRDILSTNEAL
RMAGEEGNNEVVKLLLLWKGNLHYAVIGALQGDQYDLIHKYENQIGDFHFILPLIQDAN
TFEKCHALERFCGVSCLLKHATKYNMLPILQKYQEELSMRAYLHETLFELACLWQRYDV
LKWIEQTIHVYDLKIMFNIAISKRDLTMYSLGYIFLFDRGNTEATLLTQHLKKTAAGL
LHFVLETLYGGNIDTVLTQAVKYNHRKLLDYFLRQLPRKHIEKLLLLAVQEKASKKTL
NLLLSHLNYSVKRIKKLPRYVIEYESTLVIKILLKKRVNLIIDAMLEKVMRYFSATKVRT
IMD**ELISIS**PERVIKMAIQKMRTDIVIHSTSYVWEDDLERLTRKKNMVYTIKYEHGKKMLI
KVMHGIYKNLLYGEREKVMFYLAELYVAQNAATQFRDICKDCYKLDVARFKPRFKQLIL
DCLEIITKKSCYSILEILEKHIISLFTMKVMTEEEKNLCEILYKVIHYKTIQC

Figure 3-9. IRF3 binding motif of MGF505-2R Amino acid sequence of MGF505-2R, highlighted in magenta is the IRF3 binding pLxIS motif where p indicates a hydrophilic residue.

3.3 Discussion

Using various computational methods, I have been able to gain insight into the potential role of the MGFs of interest. A consistent theme has been the predicted presence of ANK repeats, which were identified using several analysis methods. A single ANK motif is approximately 33 residues in length and consists of characteristic 2 antiparallel alpha helices connected via a perpendicular beta-hairpin or loop (Mosavi, Cammett *et al.* 2004). Although single motifs have been observed, they are typically found as numerous repeating stacked motifs. The number of repeats varies with 33 being the highest observed in a single protein, but the majority of proteins contain 6 or less (Mosavi, Cammett *et al.* 2004). Proteins containing ANK repeats have a variety of functional roles, although the common theme is that the repeating motif is crucial in protein-protein interactions with a broad spectrum of targets. It is thought that the interactions are not mediated by the specific sequence of the ANK repeat, but in fact due to the overall composition of the stacked motifs which form in a curved architecture due structural conformation of the individual motif (Mosavi, Cammett *et al.* 2004). Furthermore the ANK repeats are widely found throughout nature but not viruses, except the poxviruses (Mosavi, Cammett *et al.* 2004, Jernigan and Bordenstein 2014, Herbert, Squire *et al.* 2015). BLASTp analysis was the first to hint towards this motif, although only MGF360-10L, 12L and MGF505-3R matched with ANK proteins. HMMscan motif finder predicted the presence of the ANK motif and identified potential ANK repeats, which varied in number between different MGF proteins, from 0 in MGF360-13L and 14L, to 11 in MGF505-4R (Table 3-1). By using SWISS-MODEL to generate putative tertiary protein structure

models based on known structures of template proteins rather than only sequence homology, I added an additional method to the repertoire. SWISS-MODEL putative structural analysis also predicted the presence of ANK repeats (Figure 3-1). However only MGF360-11L, 12L and 14L created models considered to be of a “high quality”. This is likely due to lack of described proteins with a good degree of similarity to the MGF proteins, reflecting the BLASTp findings; thus, applying appropriate templates proved challenging. However almost all the models built by the software were based upon ANK containing proteins. This included Tankyrase-1 which has 5 clusters of multiple ANK repeats and was the basis for the MGF360-11L and 14L model (Eisemann, McCauley *et al.* 2016). DARPins showed similarity to all MGFs analysed. These are a class of genetically engineered antibody mimetics that utilize ANK repeats and exhibit high affinity protein-protein interactions (Plückthun 2015). Interestingly using the SWISS-Model software, the regions of the MGFs predicted to have ANK repeats approximately matched the results obtained using HMMScan where the repeating motifs were projected to start from residues 35-60 and run until close to the C-terminus. Taken together, as the results of the computational analysis point towards the presence of ANK repeats, I believe it is an area that warrants further examination. Although the predicted presence of ANK repeats alone is not sufficient to give indication of a specific potential binding partner, it does provide a theory for the general mechanism of protein-protein binding. Despite the predicted structural similarity, the lack of a well conserved sequence to known ANK containing proteins compounds the inability to identify a potential binding target. Additionally, it cannot go unsaid that there are inherent problems of using computational analysis for predicting protein structure. Without solving the exact

structure of the individual proteins, the examination is fundamentally speculative and assumes that data from alternate proteins is appropriate to construct models from. However, the computational examination provides an investigative platform to build upon.

The CRL complex is a group of cellular E3 Ub ligase machineries that catalyses the ubiquitination of target proteins for 26S proteasomal degradation (See 1.1.7.4). Cellular substrate binding F-Box proteins are one of the 5 classes of proteins capable of recruiting and acting as a platform for the CRL machinery. F-Boxes are commonly observed in poxvirus proteins and are typically associated ANK repeats, together known as PRANC motif (See 1.1.7.4).

The F-Box was not obviously observable in the in ASFV MGFs of interest, however the related VHL-Box and SOCS-Box motifs were. Due to the modular nature of recruitment of the CRL complex, there are multiple methods of recruiting the proteins required for the formation of the machinery. This includes but is not limited to the VHL-Box and SOCS-Box proteins (See 1.1.7.4) Originally found in the SOCS proteins the C-terminus, the 40 residue SOCS-Box motif consists of two distinguishable regions, termed BC-Box and Cul5-Box, along with substrate binding domains. Unlike F-Box proteins which recruit SKP1 Cul1 and RBX1 for the formation of the CRL; SOCS proteins is recruit the EloB EloC heterodimer complex via the BC-Box, and the Cul5 scaffold protein binding driven by the Cul5-Box motif, along with RBX2 (Figure 1-3) (Kamura, Sato *et al.* 1998, Zhang, Farley *et al.* 1999). Moreover, Kamura *et. al.* (1998) demonstrated the presence of a SOCS-Box-like

motif within the VHL tumour suppressor protein, which catalyses the ubiquitination of hypoxia-inducible factor α (HIF- α) (Kamura, Sato *et al.* 1998), key in the body's response to hypoxic conditions (Ziello, Jovin *et al.* 2007). This was shown to be formed of an almost identical BC-Box and additionally contains a highly similar but functionally distinct Cul binding region (known as a Cul2-Box) shown to interact with Cul2, rather than Cul5 (Kamura, Burian *et al.* 2001, Kamura, Maenaka *et al.* 2004). There appears to be a degree of promiscuity within the Cul binding motif in VHL-Box and SOCS-Boxes, as both are capable of binding Cul2 or Cul5 when over-expressed in cells (Kamura, Burian *et al.* 2001). Numerous proteins have been found to drive the formation of the E3 CRL machinery via a BC-Box coupled to an adjacent a Cul2-Box or Cul5-Box. These proteins can be broadly divided into two subgroups; VHL-Box and SOCS-Box proteins based upon whether a Cul2-Box or Cul5-Box is present (Kamura, Maenaka *et al.* 2004, Mahrour, Redwine *et al.* 2008).

By comparing the MGFs to the regions of these cellular proteins responsible for the recruitment of the CRL machinery of SOCS and VHL proteins, I was able to reveal the presence of a N-terminus motif that resembled the BC-Box and a Cul2/5-Box in the MGFs, however specifically which Cul binding region is present was unclear (Figure 3-6). MGF505 BC-Box showed a higher similarity to that of SOCS proteins, but was flanked by what appeared to resemble more of a Cul2-Box as in VHL proteins. MGF360 cluster have a motif resembling a BC-Box in a similar position to that of the MGF505 proteins, it is however missing key residues believed to be required for functional activity. The MGF360 cluster also

have what appears to be the remnants of a Cul2/5-Box, although this is further downstream from the BC-Box than in the MGF505, 22 residues compared to 2-4. However, a high degree in variation of the length of linker region in cellular proteins has also been observed (Mahrouf, Redwine *et al.* 2008). Further functional analysis determining that the motifs are functional can be found in Chapter 7.

Despite the vast majority of ANK proteins containing PRANC motifs, Odon, Georgana *et al.* (2018) reported for the first time several conserved pox proteins containing a novel C-terminus BC-Box-like motif. Furthermore, they demonstrated ectromelia virus (ECTV) 010, cowpox virus 019 had the ability to bind Cul2, despite the lack of an obvious Cul binding box. ECTV protein 010 was shown to be a strong inhibitor of the type-I IFN response via both the NF κ B and IRF3 pathways. The BC-Box alone of 010 was capable of associating with Cul2, and by using deletion mutants they showed it was responsible for the inhibitory activity (Odon, Georgana *et al.* 2018). They were unable to recognize any ANK repeat interacting partner or mechanism of inhibition of the interferon pathway. Additionally, the cowpox CP77 which is predicted to have 9 ANK repeats and has been shown to suppress the NF κ B signalling pathway, appears to have a motif that was described as a significantly truncated F-Box-like motif. The motif however would appear to match the Cul2-Box consensus sequence, but interestingly maintained the capacity to pull down Cul1, a key hallmark of the SCF driven CRL machinery. Interestingly they did not describe a BC-Box (Chang, Hsiao *et al.* 2009). Brady, Haas *et al.* (2015) showed that MC132 of *Molluscum*

Contagiosum virus targets p65 for proteasomal degradation and interacts with EloB/C and Cul5, but also that siRNA knockdown of EloB and Cul5 compromised p65 degradation. The MC132 protein has a complete BC-Box and partial Cul5-Box, however deletion of these regions did not reduce the ability to drive ubiquitination and degradation of p65. Similarly, Kaposi's sarcoma-associated herpesvirus proteins LANA1, and ORF73 of murine herpesvirus-4 have been shown to degrade p53 and NFκB respectively via SOCS-like CLR machinery (Cai, Knight *et al.* 2006, Rodrigues, Filipe *et al.* 2009). Despite the limited evidence these studies indicate that alternate CRL recruitment architecture has precedent in large DNA viruses. Interestingly HIV-1 Vif protein contains a SOCS-Box-like domain that recruits EloB, EloC, Cul5 and RBX2 allowing formation of CRL complex, but requires core binding factor B for stabilization (Zhou, Evans *et al.* 2012, Lu, Bergeron *et al.* 2013, Guo, Dong *et al.* 2014). It has been observed to interact with antiviral APOBEC3 proteins leading to their ubiquitination and subsequent proteasomal degradation (Shirakawa, Takaori-Kondo *et al.* 2006). Mutation of the BC-Box motif leads to inactivation of Vif (Kobayashi, Takaori-Kondo *et al.* 2005). This is further evidence of viral proteins utilizing CRL machinery to induce the destruction of antiviral proteins in order to aid viral survival.

The structure of the SOCS2 EloB EloC complex was solved by crystal structure at 1.9Å, revealing that the SOCS-Box is comprised of three core helices (Bullock, Debreczeni *et al.* 2006). With a helix falling at the BC-Box, followed by a second short helix, and finally the third falling immediately upstream of the LP motif of

the Cul5-Box. Together this forms a four-helix bundle with the H1 helix of EloC, with the α -helix of the BC-Box facilitating the interaction via hydrophobic residues. Subsequent interaction with EloB functions to stabilize the complex (Bullock, Debreczeni *et al.* 2006). This was also observed in SOCS3, SOCS4 and ASB proteins in complex with EloB and EloC, but importantly also in VHL (Bullock, Rodriguez *et al.* 2007, Babon, Sabo *et al.* 2008, Muniz, Guo *et al.* 2013, Nguyen, Yang *et al.* 2015). By using secondary structure prediction software Jpred, I observed a putative helix at the location of the predicted BC-Box, and another adjacent to the Cul2/5-Box in both MGF360 and 505 clusters (Figure 3-7). The lack of the second helix observed in SOCS/VHL proteins does not necessarily indicate lack of functionality of the motif in the MGFs, as it would appear there is some level of redundancy in the exact structure required. Crystal structure of HIV-1 Vif show only 2 helices are sufficient for the recruitment of the CRL complex (Guo, Dong *et al.* 2014). The related F-Box also shows a structure formed of three core helices that resembles the structure of the BC-Box (Schulman, Carrano *et al.* 2000). However it has been reported that the structure of the poxvirus' PRANC F-Box is typically truncated and despite being formed of two rather than three helices it maintains SKP1 binding ability (Sonnberg, Fleming *et al.* 2009). Overall, it would not be beyond reasonable doubt to suggest that the predicted presence of two helices in the MGFs rather than three may still allow for functionality as shown in Chapter 7.

Here it was also demonstrated that MGF360-11L, 12L have an atypical TRAF6 binding motif, and additionally MGF360-14L shows a canonical TRAF6 binding

motif (Figure 3-8). TRAF6 is an E3 Ub ligase and a key adaptor protein in the signalling pathway of the NFκB driven IFN response, by acting down stream of numerous signalling receptors (Walsh, Lee *et al.* 2015). Furthermore I also demonstrated that MGF505-2R has an IRF3 binding motif (Figure 3-9). The presence of these binding motifs hints towards a role of the MGFs as interactors with the cellular proteins. Viral protein targeting of both IRF3 and TRAF6 has been reported numerous times (Seago, Hilton *et al.* 2007, Saito, Murata *et al.* 2013, Stack, Hurst *et al.* 2013, Gottipati, Holthauzen *et al.* 2016).

Overall, here I have demonstrated that computational analysis of ASFV MGF360 and MGF505 clusters revealed a range of previously unseen motifs and architectures that provides indications of their functionality. I therefore propose that the basic structure of the MGFs is an N-terminus novel SOCS/VHL-Box approximately covering the initial 30-50 residues, with an adjacent region of ANK repeats (Figure 3-10). As it is suspected that poxvirus ANK-F-Box proteins utilize the CRL complex to target cellular substrate bound to the ANK repeat, it is not beyond reasonable doubt that the MGFs act in a similar fashion.

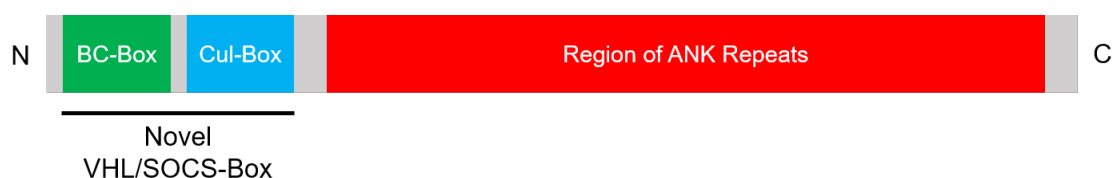


Figure 3-10 Schematic showing proposed architecture of the proteins analysed in MGF360 and MGF505 clusters

4 YEAST-2-HYBRID ANALYSIS PROBING FOR INTERACTING PARTNERS OF THE MGF360 & MGF505 PROTEINS

4.1 Introduction

ANK repeats have been extensively shown to mediate protein-protein interactions. Due to the previously described putative ANK repeats encoded within the MGF360-10L, 11L, 12L, 13L, 14L, MGF505-1R, 2R, 3R genes analysed (See Chapter 3), I proposed that the proteins may be utilising the motifs for interactions with cellular motifs. Importantly these genes have been implicated in modulation of the IFN pathway (See 1.2.4). I therefore hypothesized that the proteins may be inhibiting the IFN pathway by targeting a component of the signalling cascades and thus inhibit the type-I IFN response. Other virus proteins that inhibit this response are described in 1.1.7. I therefore set out to test protein-protein interactions between specific components of the innate signalling pathway and the MGFs of interest by utilising a targeted yeast-2-hybrid screening approach. This would allow me to rapidly test interactions between the MGFs and the selected components.

4.2 Results

4.2.1 Yeast-2-Hybrid Assay Overview

The yeast-2-hybrid assay allows testing of physical interactions between two proteins of interest, known as “prey” or “bait”. The assay utilises strains of *S. cerevisiae* genetically modified to lack the ability to biosynthesise a specific set of nutrients, thus the strains require supplementation of these nutrients for survival. This enables selection of yeast exogenously expressing genes coding for enzymes that produce the required nutrients on media lacking the corresponding nutrient, known as dropout media. Bait and prey are cloned into vectors expressing the genes required to produce differing nutrients allowing selection of successfully transfected yeast on appropriate dropout media (Figure 4-1. Step 1 and 2). The MGF genes were cloned as bait into pGBKT7 vector which expresses phosphoribosylanthranilate isomerase gene, TRP1, allowing biosynthesis of tryptophan (Table 4-1). IFN pathway components were cloned as prey into vectors expressing the beta-isopropylmalate dehydrogenase gene (LEU2) enabling the biosynthesis of leucine (Table 4-2). This allows selection of yeast transfected with vectors containing the MGFs to be selected on –Trp, and IFN components on –Leu dropout media. Bait and prey plasmids were transfected into yeast strains of opposing sex (AH109 MAT α , and Y187 MAT α respectively) (See 2.5.1), this allowed mating (See 2.5.3), resulting in yeast cells containing both plasmids to be selected on double dropout media lacking both -Trp and -Leu (Figure 4-1. Step 3 and 4). This was to improve the time efficiency and cost of carrying out the assays.

Bait	
Insert	Vector
MGF360-10L	pGBKT7
MGF360-11L	pGBKT7
MGF360-12L	pGBKT7
MGF360-13L	pGBKT7
MGF360-14L	pGBKT7
MGF505-1R	pGBKT7
MGF505-2R	pGBKT7
MGF505-3R	pGBKT7

Table 4-1. Yeast-2-Hybrid "Bait" showing insert and vector

Prey	
Insert	Vector
cGAS (porcine)	pGADT7
STING (porcine)	pGADT7
IKKε	pGADT7
IRF3	pHON3
LGP2	pGADT7
MDA5	pGADT7
SINTBAD (mouse)	pGADT7
NAPI	pGADT7
RIG-I	pGADT7
Riplet	pGADT7
TAK1	pGADT7
TANK	pGADT7
TBK1	pGADT7
TRAF3	pGADT7
TRAF6	pGADT7
TRIF	pGADT7
TRIM25	pGADT7
MAVS	pGADT7

Table 4-2 Yeast-2-Hybrid "Prey" showing insert and vector cGAS and STING were cloned in to pGADT7 from porcine macrophages infected with ASFV isolate OURT88/3.

The assay for interacting proteins utilises activation of downstream reporter genes (e.g. HIS3, MEL1) to test for positive interactions as described in 2.5.4. Bait proteins are expressed fused to Gal4 binding domain which binds to the upstream activation sequence (UAS) of the reporter genes. Prey proteins are expressed fused to a Gal4 activation domain. If there is no interaction between the bait and prey, there is no expression of the reporter genes (Figure 4-2A). On interaction of the two proteins, the prey comes into proximity to the bait bound to the UAS. This effectively reconstitutes the Gal4 transcription factor, in turn activating transcription of the reporter genes (Figure 4-2B). Interaction of the bait and prey leads to the additional expression of HIS3 and ADE2, enabling yeast survival on -Trp, -Leu, -His and -Ade dropout media. The MEL1 gene expressing galactosidase is also activated allowing a colorimetric assay in which colonies turn blue in the presence of X- α -Gal (Figure 4-1. Step 5). Therefore, yeast transfected with two plasmids expressing interacting proteins would be selected by growth on a quadruple dropout media (-Leu, -Trp, -His, -Ade) and colonies would turn blue in the presence of X- α -Gal.

The yeast-2-hybrid assay allows a rapid, cost effective and highly sensitive method of detecting protein-protein interactions, there are however a number of caveats with the assay which are discussed later.

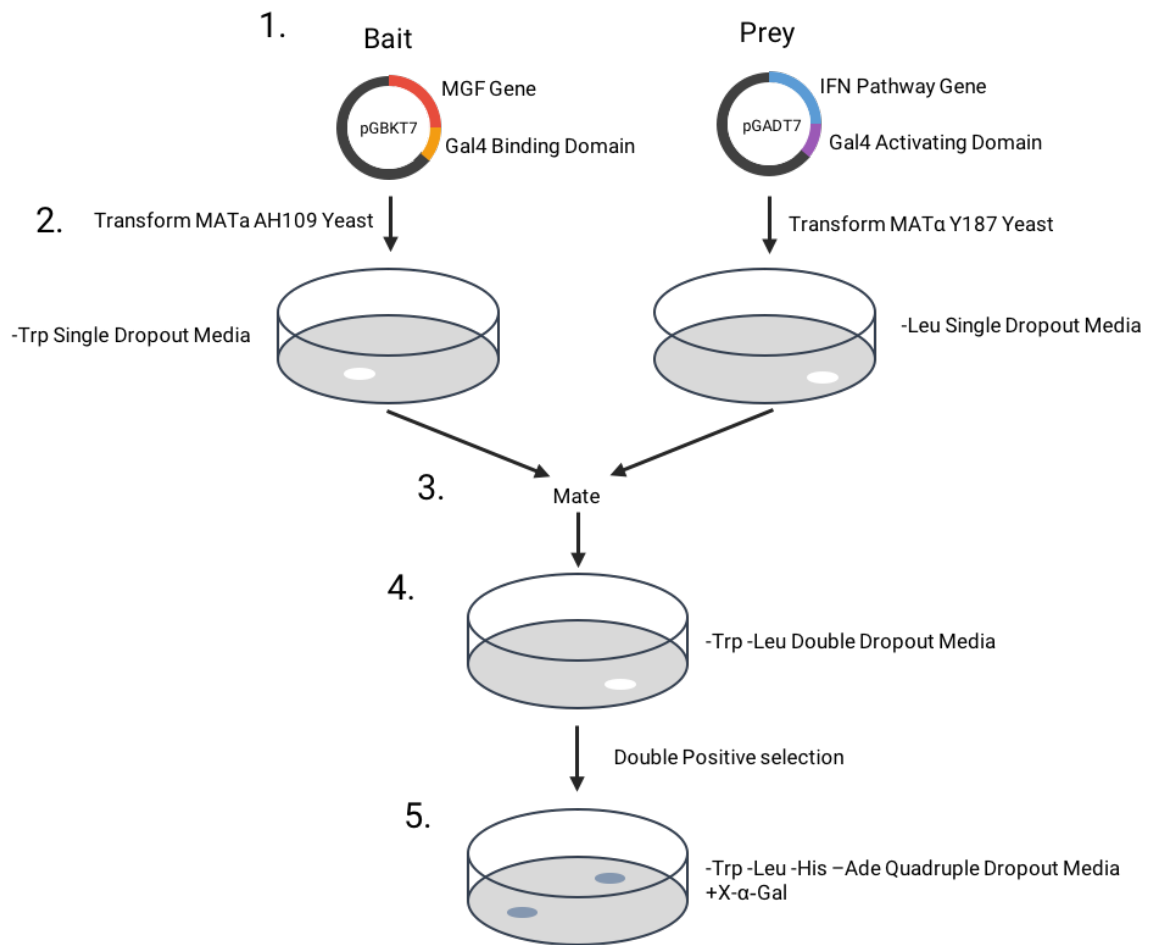
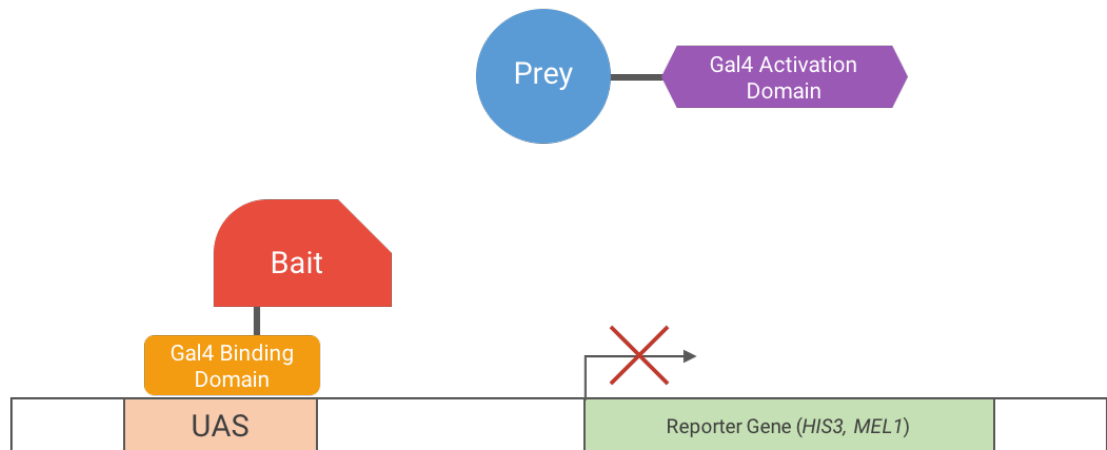


Figure 4-1. Overview of the critical stages of the yeast-2-hybrid assay 1. Describes plasmids vectors containing bait and prey with corresponding selective markers. 2. Transformation and selection on single dropout media. 3. Mating of yeast. 4. Selection of successfully mated yeast containing both plasmids. 5. Selection of positive interactors on quadruple dropout media in the presence of X-α-Gal. Note the blue colouration of colonies.

A.



B.

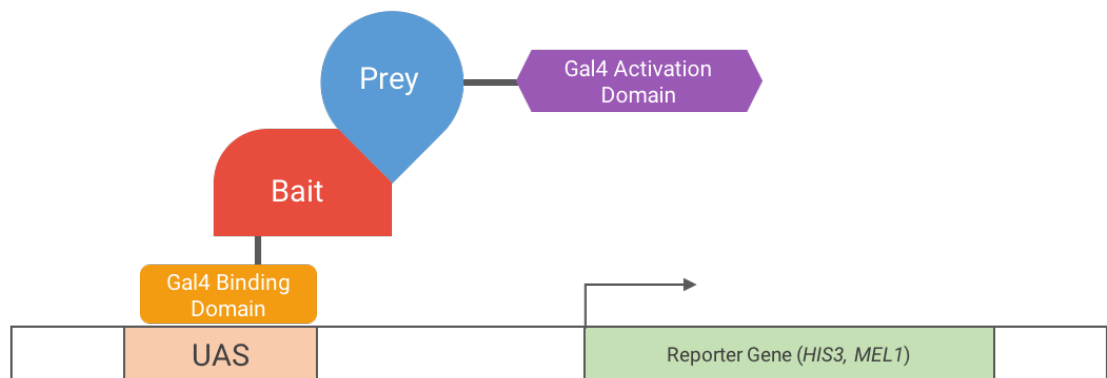


Figure 4-2. Key principle of the yeast-2-hybrid assay **A.** Shows no interaction between bait and prey, thus no induction of the expression of the reporter genes. **B.** However shows the interaction between bait and prey bringing the Gal4 activation domain into to proximity to the reporter gene leading to transcription.

4.2.2 MGF Constructs were Tested for Self-Activation

pGBKT7 with inserts of MGF360-10L, 11L, 12L, 13L, 14L, and MGF505-1R, 2R, 3R were tested for self-activation by “leaky” His expression. “Leaky” His expression is a low level background expression of HIS3 that is known to be induced by some bait constructs. If this were to occur, the low level HIS3 expression would allow growth on media deficient in His, leading to false positive results. This can however be mitigated by the presence of 3AT, a competitive inhibitor of the HIS3 enzymatic product, therefore raising the stringency for growth on media deficient in His. To test for leaky HIS3 expression, yeast transfected with the pGBKT7.MGF360s or pGBKT7.MGF505s were grown on –His single dropout media supplemented with an increasing concentration of 3AT (0 mM, 5 mM, 10 mM, 15 mM, 20 mM). Yeast transfected with pGBKT7 expressing MGF360-11L, 14L, MGF505-3R displayed growth on –His single dropout media, with 0 mM, and 5 mM 3AT, but not on any higher concentrations. Therefore, any yeast-2-hybrid assays investigating interactions between prey and MGF360-11L, 14L, or MGF505-3R were performed in the presence of 10 mM 3AT to eliminate this “leaky” HIS3 expression giving a false positive result.

4.2.3 MGF360-11L, 12L, 13L & MGF505-3R Interacted with TRAF6

Using the approach described in 2.5.4, MGF360-11L, 12L, 13L, and MGF505-3R displayed positive interactions with TRAF6. I observed growth of transfected and successfully mated colonies on quadruple dropout media, but also a strong blue colouration in the presence of X- α -Gal. Furthermore all positive interactions were

capable of growth in the presence of up to 15mM of 3AT, indicating a reasonably strong interaction between bait and prey (Figure 4-3).

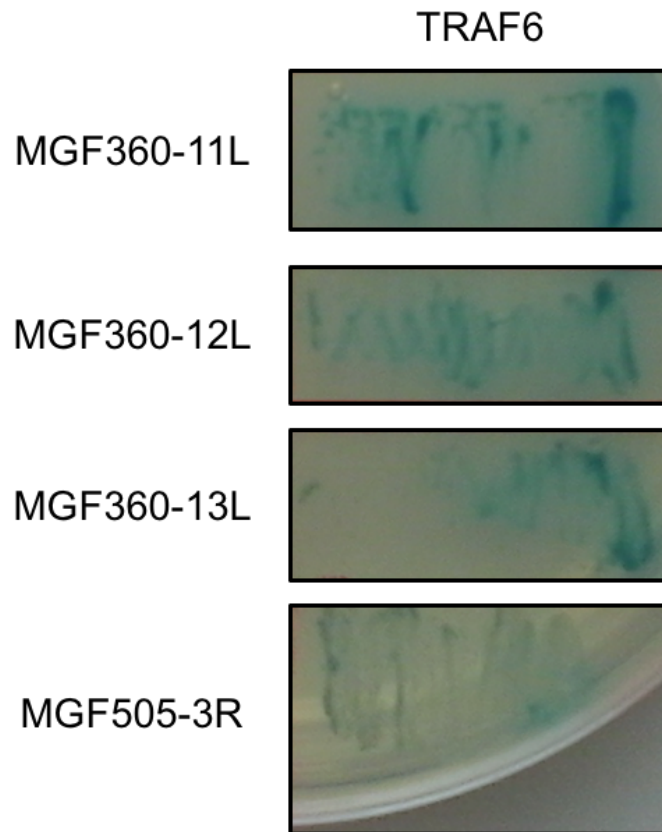


Figure 4-3. Interactors of TRAF6 Yeast cells were transfected and mated to contain constructs expressing MGFs and TRAF6 as bait and prey, respectively. Interactions between the two were tested by growth on selective dropout media in the presence of colorimetric reporter X- α -Gal. Blue colonies indicate a positive interaction between pGBKT7.MGF360-11L, 12L, 13L, and MGF505-3R with pGADT7.TRAF6.

4.2.4 MGF360-12L Interacted with TRAF3

Successful growth of colonies assessing interactions between MGF360-12L and TRAF3 was observed on quadruple dropout media, and these colonies developed a blue colouration in the presence of X- α -Gal (Figure 4-4). This positive result therefore indicated a putative interaction between MGF360-12L and TRAF3, a component in the transduction of the IFN pathway via leading to the activation of IRF3 and to a lesser extent NF κ B (Häcker, Redecke *et al.* 2006, Fang, Jiang *et al.* 2017).

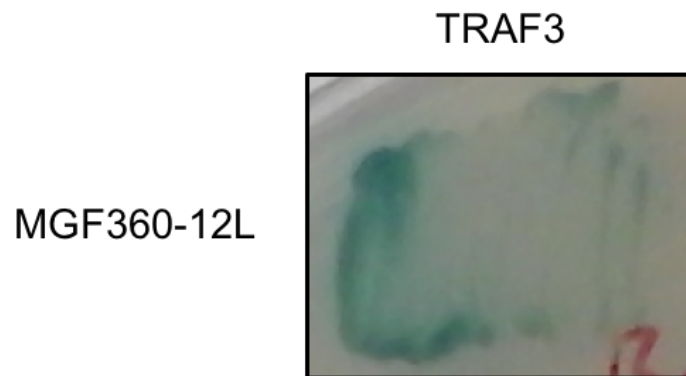


Figure 4-4. MGF360-12L interacts with TRAF3 Yeast cells were transfected and mated to contain constructs expressing MGFs and TRAF3 as bait and prey, respectively. Interactions between the two were tested by growth on selective dropout media in the presence of colorimetric reporter X- α -Gal. Colonies indicating positive interaction between pGBKT7.MGF360-12L with pGADT7.TRAF3.

4.2.5 MGF360-13L Interacted with IRF3

A positive interaction was observed between MGF360-13L and key IFN transcription factor IRF3, on quadruple dropout media, with blue colouration in the presence of X- α -Gal (Figure 4-5). This was performed at 15mM with HIS3 product competitive inhibitor 3AT, indicating a fairly strong interaction.

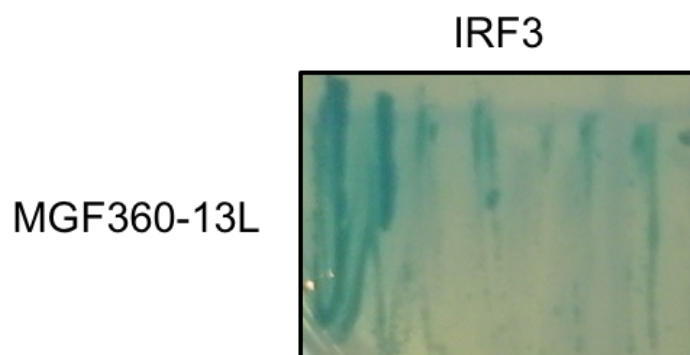


Figure 4-5. MGF360-13L interacts with IRF3 Yeast cells were transfected and mated to contain constructs expressing MGFs and IRF3 as bait and prey, respectively. Interactions between the two were tested by growth on selective dropout media in the presence of colorimetric reporter X- α -Gal. Colonies indicating positive interaction between pGBKT7.MGF360-13L with pHON3.IRF3.

4.3 Discussion

Here I have demonstrated interactions between MGF360-11L, 12L, 13L, and MGF505-3R with cellular proteins TRAF3, TRAF6 and IRF3 by yeast-2-hybrid assay. Each of these are considered critical components in the IFN induction pathway. If the interactions are confirmed these potentially provide great insight into the molecular function of the MGF genes. Overview of the findings can be seen in Table 4-3.

MGF	Interactor
MGF360-11L	TRAF6
MGF360-12L	TRAF3, TRAF6
MGF360-13L	TRAF6, IRF3
MGF505-3R	TRAF6

Table 4-3. Overview of the potential interactions between MGFs and components of the IFN signalling pathway

As I had planned to undertake a targeted yeast-2-hybrid approach, where the interactions of bait MGFs were assessed against specific components of the IFN pathway, as opposed to a library, the profile of IFN components tested was given careful consideration. I effectively divided the IFN response into two areas, stimulation of the signalling pathway leading to either NFκB or IRF3 activation. Activation of PRRs triggers a downstream signalling cascade that ultimately ends in the activation of transcription factors such as NFκB or IRF3 where they translocate to the nucleus to induce IFN expression (See 1.1.4). Components tested were selected to cover key junctions of each arm of the response. This included but was not limited to components involved in initial activation of the

signalling cascade (PRRs - cGAS and RIG-I), key proteins involved in the transduction of the signal (e.g TRAF3, TRAF6, TBK1, IKK ϵ), and finally basal level transcription factors (e.g. IRF3), amongst others that are important in the induction of IFN (e.g. Riplet and tripartite motif containing protein 25 (TRIM25)). Due to the fact that ASFV is a large DNA virus, porcine DNA receptor cGAS and STING were cloned in the prey pGADT7 vector as it would appear to be a logical step within the pathway for the virus to inhibit. However, I did not observe any interactions of the MGF proteins with either proteins. Despite possessing a prey construct expressing p65 (a component of the NF κ B transcription factor), I was unable to successfully transform yeast with this plasmid. This may be due to p65 having a stimulatory effect within the yeast cells. Indeed, p65 was shown to be recognised by yeast transcription machinery, and its overexpression is consequently toxic for the cells (Epinat, Whiteside *et al.* 1997).

Here I demonstrated that MGF360-12L interacted with TRAF3, and TRAF6. Whereas MGF360-11L, 13L, and MGF505-3R interacted with TRAF6 alone. The TRAFs are a family of seven proteins (TRAF1-7) involved in controlling signal transduction initiated by PRRs, including TLRs, or RLRs, and IL1R leading to an innate immune response. The family of proteins (except TRAF1) exhibit a modular structure with a well characterised C-terminus coiled coil TRAF domain that induces oligomerisation facilitating interactions with other proteins of the signalling cascades; additionally TRAFs including TRAF3 and TRAF6 function as E3 Ub ligases (Häcker, Tseng *et al.* 2011, Park 2018). Both TRAF3 and TRAF6 E3 Ub ligase activity is mediated by a RING finger motif, conferring their ability to

generate Lys63 linked poly-Ub chains via the recruitment of E2 ligase proteins such as Ubc5, Ubc13 or Uev1A (Deng, Wang *et al.* 2000, Wang, Deng *et al.* 2001). This Ub activity is key in the modulation of the IFN signalling pathway. For example, ubiquitination by TRAF6 was shown to be crucial in the NFκB response, with K63 polyubiquitination of itself and IKKγ required for their activation (Deng, Wang *et al.* 2000, Sun, Deng *et al.* 2004).

It is of note that despite functional and structural similarities between TRAF3 and TRAF6, the two appear to play distinct roles in the IFN signalling cascade (Figure 4-6 and Figure 4-7). Both have been shown to induce a strong IFN response, with TRAF3 mostly directing this via the IRF3 pathway, whereas TRAF6 acts mainly via the NFκB pathway, although there is some overlap and cross-pathway communication (Häcker, Redecke *et al.* 2006, Oganessian, Saha *et al.* 2006, Fang, Jiang *et al.* 2017). TLRs initiate signalling cascades by recruitment of TIR domain containing adaptor molecules, such as MyD88, and TRIF to their cytoplasmic tails (See 1.1.5.1). The variation in function between TRAF3 and TRAF6 is partially due to interaction with the TIR domain of differing TLR adaptor molecules. TRAF3 can interact with both MyD88 and TRIF, however it is the interaction with TRIF that directs the cascade to activate TBK1 and thus IRF3 (Häcker, Redecke *et al.* 2006). TRAF3 is thought to play a role in the canonical NFκB pathway albeit to a lesser extent, whereas this pathway is the focus of TRAF6. By interacting with the TIR domain of MyD88 TRAF6 is essential for the subsequent signalling cascade and canonical NFκB activation, but as it does not interact with TRIF, is thus expendable for TRIF mediated IRF3 and NFκB signalling (Gohda, Matsumura *et*

al. 2004, Häcker, Redecke *et al.* 2006). Furthermore, Konno, Yamamoto *et al.* (2009) demonstrated that the NFκB and IRF7 responses to RNA virus infection were significantly impaired in the absence of TRAF6, whereas the IRF3 response was not.

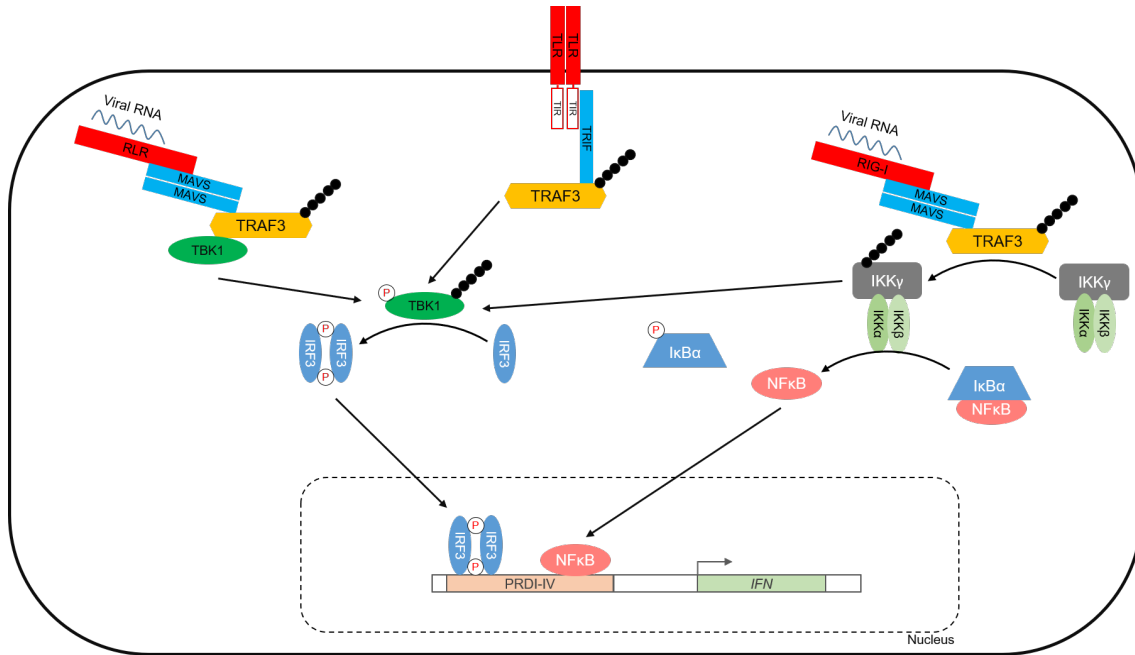


Figure 4-6. The role of TRAF3 in the signalling pathways leading to IFN expression

Overview of the role of TRAF3 in IRF3 and NFκB activation. Despite predominantly stimulating the TBK1-IRF3 pathway, the right-hand section shows how TRAF3 is also capable of inducing NFκB activation via MAVS-IKK. Black circles indicate poly-Ub chains. Red P indicates phosphorylation.

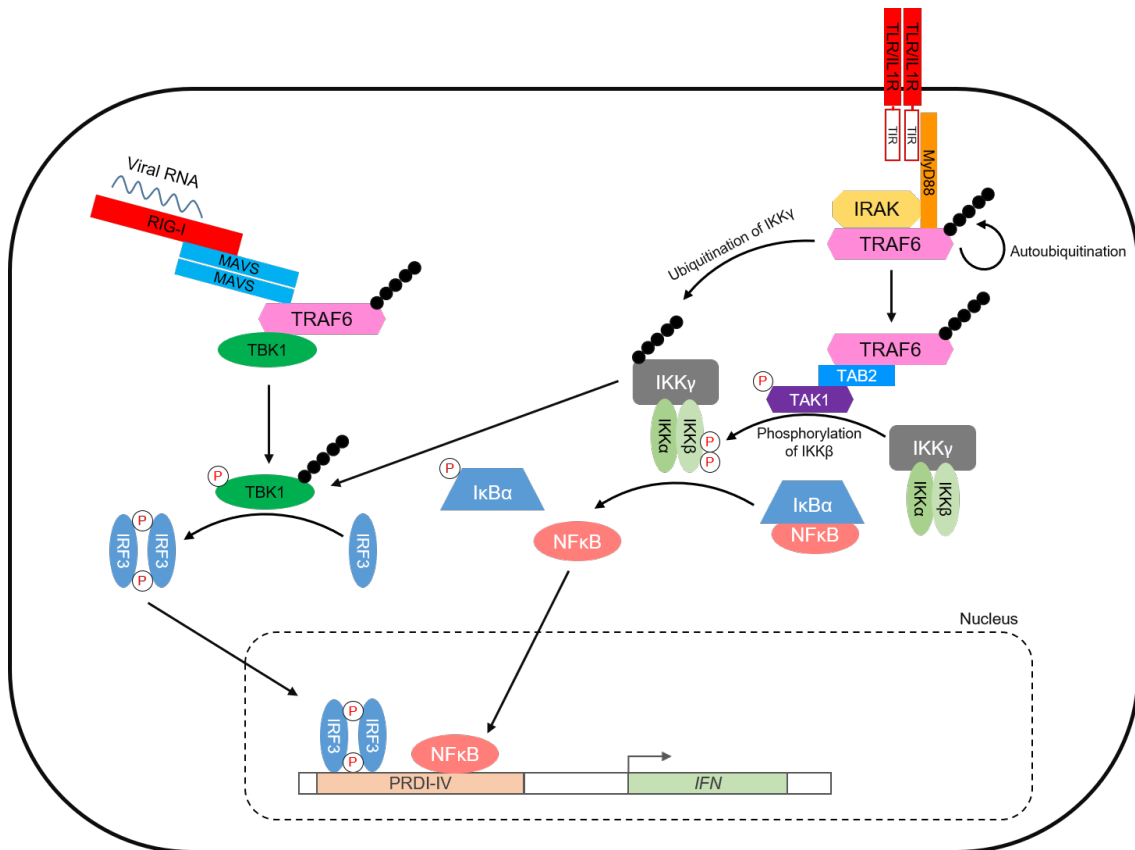


Figure 4-7. The role of TRAF6 in the signalling pathways leading to IFN expression

Overview of the role of TRAF3 in IRF3 and NFκB activation. Despite predominantly stimulating the NFκB pathway, the left-hand section shows how TRAF6 is also capable of inducing IRF3 activation via bringing TBK1 into proximity to MAVS allowing ubiquitination and phosphorylation by IKK. This therefore demonstrates a level of crossover between the two signalling pathways. Black circles indicate poly-Ub chains. Red P indicates phosphorylation.

Importantly TRAF6 has been shown to be necessary in IKK activation. TRAF6 mediated non-degradative polyubiquitination via the recruitment of Ubc13/Uev1A E2 ligases at Lys63 activates IKK complex by forming a Ub chain on IKK γ . This subsequently results in the degradation of I κ B α and thus NF κ B activation (Deng, Wang *et al.* 2000, Sun, Deng *et al.* 2004). Furthermore, TRAF6 associates with IRAKs and following IL-1 β stimulation forms a complex with the downstream MAP kinase kinase TAK1 thus activating it to allow phosphorylation of IKK β and eventually NF κ B activation (Jiang, Ninomiya-Tsuji *et al.* 2002).

The overlap between TRAF3 and TRAF6 appears to be due to their influence on the PRR RIG-I initiated pathway via MAVS. Following RLR stimulation via recognition of viral RNA, TRAF3 associates with MAVS leading to its non-degradative Lys63 polyubiquitination, and recruitment of TBK1, leading to an IRF3 response (Paz, Vilasco *et al.* 2011, Fang, Jiang *et al.* 2017). Likewise Fang, Jiang *et al.* (2017) reported that TRAF3 and TRAF6 activation of NF κ B occurs via MAVS. MAVS mediated TRAF3 and TRAF6 E3 ligase activity drives activation of IKK γ by the addition of poly-Ub chains which are required for activation of IKK α/β and subsequent NF κ B activation. Both these TRAFs have been shown to stimulate both IRF3 and NF κ B pathways via MAVS in an IKK γ dependant and independent manner. Independently of IKK γ , TRAFs have been shown to recruit the closely related kinases TBK1 and IKK ϵ , by bringing the kinases in proximity to MAVS leads to their phosphorylation and activation (Fang, Jiang *et al.* 2017). Interestingly, Fang, Jiang *et al.* (2017) also reported that the MAVS-TRAFs platform unexpectedly led to the activation of phosphorylation of TBK1 in an IKK γ

dependent fashion. Although TRAF6 was previously not thought to influence RLR driven IRF3 signalling (Konno, Yamamoto *et al.* 2009), Fang, Jiang *et al.* (2017) demonstrated that both TRAF3, and TRAF6 knockout cell lines lost their ability to respond to Sendai virus infection via NFκB or IRF3 activation, but their reconstitution restored activation. Remarkably this RIG-I-MAVS-TBK1-IRF3 pathway was dependant on a variety of TRAFs but independent of their E3 ligase activity; the authors proposed that the TRAF family may form hetero-oligomers to highly refine the response to viral stimulation.

Overall, it is abundantly clear that both TRAF3 and TRAF6 play important but varied roles in induction of IFN via the IRF3 or NFκB signalling pathways. Therefore, it would not be surprising if the MGF360-11L, 12L, 13L and MGF505-3Rs were targeting the TRAFs to inhibit the IFN response. Nonetheless, the TRAFs are not seemingly common targets for viral proteins, this may be due to the proposed overlapping functionality within the family. However, it is therefore interesting a number of the MGFs showed positive interactions with TRAF3 and TRAF6; as the TRAFs contain shared architecture across the family, this may hint towards a target common to these MGFs (Figure 4-3 and Figure 4-4). Assessing potential interactions with other members of the TRAF family would be of interest. The presence of a novel TRAF6 binding motif [RxExxAr/Ac] (where Ar is an aromatic, and Ac is an acidic residue) in MGF360-11L and 12L (Figure 3-8) adds weight to the observed interaction. However, it is curious that MGF360-14L which was the sole member to have the canonical TRAF6 binding motif PxExxAr/Ac (Figure 3-8) did not yield a positive interaction. MGF360-13L and

MGF505-3R were also capable of interacting with TRAF6 despite the lack of an obvious TRAF6 binding motif. Interestingly MGF360-13L does possess the motif's core PxE residues at position 326-328. Mutation of these core residues in IL-3 and IL-5 receptors required for TRAF6 binding has been shown to be sufficient to block TRAF6 binding, whereas the Ar/Ac residue is thought to have less significance (Ye, Arron *et al.* 2002, Meads, Li *et al.* 2010). Therefore, the core residues alone may be sufficient to induce TRAF6 binding, potentially explaining our observation. MGF360-12L does not appear to have a TRAF3 binding motif (P-x-[QE]-E). However, there is a large deal of promiscuity with the motif, which is also capable of binding TRAF1, 2, and 5. It would therefore be of interest to further examine MGF360-12L's binding capacity to the additional TRAFs (Meads, Li *et al.* 2010).

MGF360-13L interaction with IRF3 was another interesting result. IRF3 is key transcription factor in the expression of type-I IFN (See 1.1.4.1). Adaptor proteins MAVS, STING or TRIF mediate the recruitment of IRF3 via an pLxIS motif (where p is a hydrophilic residue, S is a phosphorylation site) bringing TBK1 into proximity of IRF3 which leads to its phosphorylation and activation of the transcription factor (Liu, Cai *et al.* 2015). Moreover post-activation dimerisation of IRF3 occurs at an pLxIS motif within the transcription factor itself (Zhao, Shu *et al.* 2016). Numerous viral proteins inhibit the IFN pathway at the basal transcription factor level by targeting IRF3 directly (See 1.1.7.1). Rotavirus NSP1, for example, utilises a pLxIS motif to bind to IRF3 targeting it for degradation (Zhao, Shu *et al.* 2016). Interestingly MGF360-13L does not contain the pLxIS

motif, whereas MGF505-2R does (Figure 3-9) but did not show an interaction within the yeast-2-hybrid assay. Attenuation of the IFN response by targeting IRF3 has precedence in a number of viral proteins and would not be a surprising target for MGF proteins (Seago, Hilton *et al.* 2007, Randall and Goodbourn 2008, Gottipati, Holthauzen *et al.* 2016, Zhao, Shu *et al.* 2016). Further work to assess MGF360-13L's impact on IRF3 itself is required to understand the potential mechanism in more detail, including looking at inhibition of the IRF3 pathway itself and any effects on nuclear translocation of the transcription factor.

The yeast-2-hybrid assay offers a relatively rapid and low-cost method of assessing potential protein-protein interactions *in vivo* in what is arguably close to a physiologically representative environment. However, like all assays it is not without shortcomings. Interactions must occur in the nucleus for the transcription machinery to be present. But also, the fusion hybrid between the Gal4 DBD (Gal4DBD) and bait protein must not activate the reporter genes independently of the prey-Gal4AD construct. This control assay was carried out in our experiments in 4.2.2. The assay may yield false positives due to the overexpression of exogenous proteins essentially artificially forcing the interaction. It may also miss true positives however due to the absence of potential additional cellular chaperones or modifiers required for the interactions not being present in yeast.

Furthermore, by choosing to use a targeted approach risked missing prey proteins outside the pool selected of proteins that may have interacted with the

MGFs. Using a stable isotope labelling with amino acids in cell culture (SILAC) mass spectrometry approach would have alleviated this issue and potentially revealed further interacting partners. However, as I demonstrated the MGFs potential in recruitment of CLR machinery in (Figure 3-6), using a yeast system may play to our advantage. *S. cerevisiae* does possess SCF CRL homologue that is required for cell cycle progression, but not thought to have a conserved Cul2 or Cul5, which are required for the construction of the VHL or SOCS CRL (Willems, Schwab *et al.* 2004). In the absence of the appropriate CRL homologues in yeast, target proteins bound to the MGFs may not be rapidly degraded allowing observation of the interaction.

Overall, the interaction of MGF360-11L, 12L, 13L, and MGF505-3R with TRAF3, MGF360-12L with TRAF3/6, and MGF360-13L with IRF3 all offer very interesting research pathways to follow. The MGFs may be inhibiting the IFN pathway by interacting and sequestering the activity of the interaction partners. However further work to confirm these interactions is required for example by CoIP.

5 INHIBITION OF THE NF κ B SIGNALLING PATHWAY BY MGF360 & MGF505 PROTEINS

5.1 Introduction

The combined effect of deletion of MGF360-10L, 11L, 12L, 13L, 14L, and MGF505-1R, 2R and 3R on the inhibition of IFN response has been previously demonstrated (Afonso, Piccone *et al.* 2004, Golding, Goatley *et al.* 2016, Reis, Abrams *et al.* 2016). However, the functional mechanisms of individual MGF's are yet to be studied with the exception of very recent work in to MGF360-12L (See 1.2.4). Therefore, a key component of my work was to characterise the IFN inhibitory mechanisms of individual MGF genes. The work demonstrated so far here has pointed towards a prospective mechanism of action for the inhibition of the IFN pathway by the MGF proteins. I have hypothesised that the ANK repeat motifs bind to target proteins of the IFN signalling pathway, bringing them into proximity of CLR E3 ligase machinery, ultimately leading to their proteasomal degradation (See Chapter 3). I have also demonstrated potential interacting partners by yeast-2-hybrid, including TRAF3, TRAF6 and IRF3 (See Chapter 4). It was therefore a logical step to assess the inhibitory potential of the genes on the innate response and try to characterise the specific location of inhibition within the pathways.

Stimulation of the innate signalling pathway leads to the activation of transcription factors including NF κ B (See Chapter 1.1.4.2, Figure 1-2, and Figure

5-1). NFκB transcription factor is homodimer or heterodimer formed of a class of proteins which share a Rel domain, including p50, p52, p65 (RELA), c-REL, and RELB (Li and Verma 2002, Oeckinghaus and Ghosh 2009, Israël 2010). Here I will focus on the p50 p65 NFκB heterodimer, which is implicated in the canonical pathway (See 1.1.4.2). Other variants of the transcription factor heterodimer include p52 RELB which is activated following the processing of p100 to p50, and function in the non-canonical NFκB pathway (Hoesel and Schmid 2013). Activated NFκB shuttles in and out of the nucleus and has been found to bind to promoters of hundreds of genes, including type-I IFN, numerous chemokines and cytokines (Oeckinghaus and Ghosh 2009, Yang Yang 2016). In the IFN promoter, NFκB preferentially binds to sequence specific DNA known as the PRDII regulatory element (Chen, Sengchanthalangsy *et al.* 2000, Schreiber, Jenner *et al.* 2006). Overall, it is clear that NFκB is a key player in the response to viral pathogens and the induction of the innate immune response.

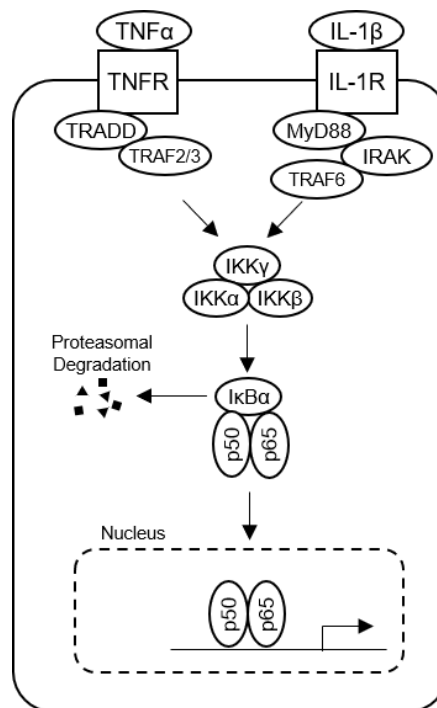


Figure 5-1. Schematic giving simple overview of activation of the NFκB pathway

Activation of the canonical NF κ B signalling pathway can be broadly divided stimulation by cytokines such as TNFR by TNF α and IL1R by IL-1 β , or PRR activation by relevant PAMPs (See 1.1.5). TNFR stimulation by TNF α recruits TNFR associated death domain, receptor interacting protein 1 (RIP1), and TRAF2/5 promoting the polyubiquitination of RIP1, forming what is known as complex 1 (Hayden and Ghosh 2014). This in turn recruits and stabilises the heterotrimeric IKK complex (IKK $\alpha/\beta/\gamma$), but also brings in proximity TAK1 which is thought to phosphorylate and activate IKK β (Hsu, Huang *et al.* 1996, Jackson-Bernitsas, Ichikawa *et al.* 2007, Hayden and Ghosh 2014). In addition, IL1R and TLRs (except TLR3) contain a TIR domain which interacts with cytosolic adaptor protein MyD88 and recruits IRAK1/4 along with TRAF6 to drive NF κ B activation (Cao, Xiong *et al.* 1996, Jefferies, Bowie *et al.* 2001, Conze, Wu *et al.* 2008, Lin, Lo *et al.* 2010, Kawasaki and Kawai 2014). This interaction promotes TRAF6 K63 auto-polyubiquitination, in addition to ubiquitination of TAK1. These Ub chains facilitate the binding of regulatory complexes of TAB1, TAB2, and TAB3 to TRAF6 to activate TAK1. Active TAK1 in turn binds to the IKK complex to phosphoactivate IKK β (Wang, Deng *et al.* 2001, Jiang, Ninomiya-Tsuji *et al.* 2002, Lin, Lo *et al.* 2010, Kawasaki and Kawai 2014). In contrast TLR3 associates with the multifunctional protein TRIF via its TIR domain, to interact with TRAF2/3/6 inducing RIP1 activation, and this in turn activates TAK1 complex via TAK1-binding protein (TAB) complex and consequently leading to IKK activation and eventually NF κ B activation (Suzuki, Suzuki *et al.* 2002, Yamamoto, Sato *et al.* 2003, Meylan, Burns *et al.* 2004, Cusson-Hermance, Khurana *et al.* 2005, Sasai, Tatematsu *et al.* 2010). Furthermore, cytosolic PRRs have been implicated in

NF κ B signalling via MAVS and STING mediated TRAF6 driven activation of the IKK complex (See Chapter 1.1.5).

Despite a high level of similarity between IKK α and IKK β and both belonging to the IKK complex, their enzymatic functions are varied with differing substrate specificities. IKK β is thought to be the primary driver of rapid I κ B α degradation which occurs in minutes (Häcker and Karin 2006), whereas IKK α was shown to be nonessential in NF κ B activation (Hu, Baud *et al.* 1999). However both kinases have been reported to activate the transcription by inducing phosphorylation of I κ B α (Karin and Ben-Neriah 2000, Adli, Merkhofer *et al.* 2010). A further two kinases with a high level of similarity to IKK α / β have been discovered, TBK1 and IKK ϵ . Although implicated in NF κ B signalling, these kinases are primarily involved in the IRF3 signalling pathway (Peters, Liao *et al.* 2000, Tojima, Fujimoto *et al.* 2000, Fitzgerald, McWhirter *et al.* 2003). Inactive NF κ B accumulates in the cytoplasm in complex with I κ B α . This negative regulator of NF κ B contains a region rich in proline, glutamic acid, serine and threonine residues known as a PEST motif present in proteins with a high proteasomal turn over (Pando and Verma 2000, Belizario, Alves *et al.* 2008), but also 6 ANK repeats which directly bind to the transcription factor (Huxford, Huang *et al.* 1998). Active IKK complex phosphorylates and activates the negative regulator I κ B α in at S32 and S36 (Traenckner, Pahl *et al.* 1995, Karin and Ben-Neriah 2000, Israël 2010). This subsequently releases the sequestered NF κ B from the negative regulator, revealing a site for ubiquitination leading to rapid I κ B α proteasomal degradation, allowing activated NF κ B to translocate to the nucleus (Huxford, Huang *et al.*

1998, Karin and Ben-Neriah 2000, Chen 2005). It was initially proposed that inhibition of NFκB by IκBα was due to masking of a nuclear localisation sequence, however it has since been shown that the binding of IκBα to NFκB induces a 180° rotational conformational change locking the transcription factor into a “closed” structure relative to its DNA binding “open” state (Huxford, Huang *et al.* 1998). Furthermore, release of IκBα allows phosphorylation of the p65 subunit in the transactivation domain (TAD) at residue S536 by kinases IKKα/β and is critical for functionality (Karin and Ben-Neriah 2000, Sakurai, Suzuki *et al.* 2003, Buss, Dörrie *et al.* 2004, Adli, Merkhofer *et al.* 2010). Interestingly phosphorylation of different residues of p65 has differing functional outcomes (Buss, Dörrie *et al.* 2004, Schmitz, Mattioli *et al.* 2004). Once activated, NFκB is then capable of binding DNA sequence specific promoter regions known as κB response elements, enhancing the expression of numerous different chemokines, cytokines, and proteins essential in the immune response, but also aids regulation of cell cycle (Schreiber, Jenner *et al.* 2006, Yang Yang 2016). Interestingly, NFκB also induces expression of negative regulator IκBα (de Martin, Vanhove *et al.* 1993). This leads to IκBα accumulating in the nucleus at a faster rate than NFκB, acting to dissociate active NFκB from the DNA response elements by inducing a conformational change as described above (Birbach, Gold *et al.* 2002, Bergqvist, Alverdi *et al.* 2009). This acts as a balance between both active and inactive NFκB, resulting in its shuttling in and out of the nucleus, thus acting as a mechanism of control over the pathway (Birbach, Gold *et al.* 2002).

Here I have investigated the ability of the MGFs of interest to inhibit the NF κ B pathway by using a variety of different techniques including luciferase assays coupled with confocal microscopy, and western blotting.

5.2 Results

5.2.1 MGF360 & MGF505 Inhibited the NF κ B Pathway at the Transcription Factor Level or Below

Using a dual luciferase assay system as described in 2.6, I investigated inhibition of the NF κ B pathway by different proteins of the MGF family. By systematically probing the pathway, I was able to determine the specific level of inhibition. This provided evidence that inhibition was occurring at the transcription factor level or below, for all MGFs proteins tested. The dual luciferase assay utilises HEK293T cells transfected with a construct containing firefly luciferase under the control of the NF κ B response element region (PRDII) of the IFN β promoter. Therefore, stimulation of the NF κ B signalling pathway would activate the transcription factor in turn inducing expression of firefly luciferase, an enzyme that catalyses a measurable bioluminescence reaction, thus one can make inferences on the activity of the pathway. Co-transfection with a plasmid construct expressing renilla luciferase provided a transfection control. Cells were further co-transfected with constructs expressing the MGF proteins, or controls including a known inhibitor of the NF κ B pathway, MC160, or empty vector. Sequences of the MGFs of interest were codon optimised for expression in human cells and encoded to express a V5-tag with the sequence,

GKPIPNNPLLGLDST fused at the N-terminus (See 2.4.5). Therefore, on stimulation of the pathway a reduction in firefly luciferase bioluminescence compared to the empty vector, can be inferred as inhibition of the pathway.

To probe the pathway, I began by stimulating from the initial point of activation using TNF α (20 ng.mL) or IL-1 β (10 ng.mL) to activate the TNF α receptor and IL-1 β receptor, respectively. Unstimulated cells received equal volume of vehicle. This revealed that all MGFs showed strong inhibition of the pathways (Figure 5-2A). MC160, a strong inhibitor of the NF κ B pathway was used as a positive control (Nichols and Shisler 2006, Nichols and Shisler 2009). Further systematic probing of the pathway was undertaken with the exogenous expression of key components (IKK α , IKK β and p65-GFP) in order to attempt to determine the specific location of inhibition. Unstimulated cells were transfected with empty vector DNA at an equal volume to the stimulant plasmid. I exogenously expressed IKK α and IKK β , both containing a Flag-tag (sequence DYKDDDDK) fused at the N-terminus. These are key kinases in the phosphorylation of I κ B α , which leads to release and activation of NF κ B transcription factor (Karin and Ben-Neriah 2000, Adli, Merkhofer *et al.* 2010). Here significant inhibition was also observed with all MGFs, hinting towards the inhibition occurring at the kinase level or below (Figure 5-2B). Finally, all MGFs tested significantly inhibited activation caused by transient expression of p65 fused to GFP (p65.GFP) construct. This suggested that the inhibition by the MGFs was occurring at the p65 level, or below (Figure 5-2C). Expression of p65.GFP and flag.IKK β , MGFs, MC160 were confirmed by western blotting (Figure 5-2D and E).

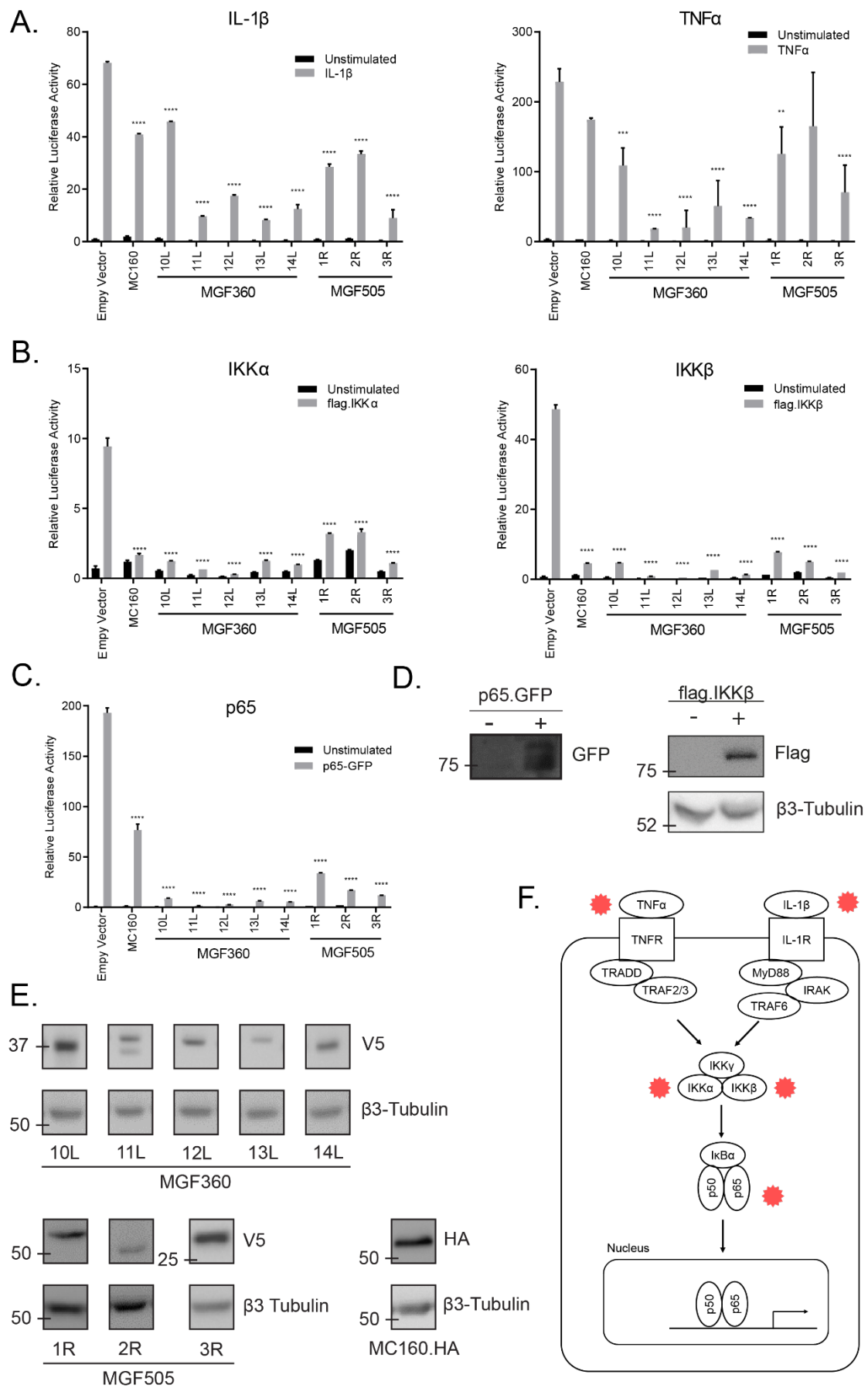


Figure 5-2. Luciferase data showing assessment of the MGFs inhibition of the NFκB pathway HEK293T cells were transfected with luciferase reporter gene under the control of NFκB regulatory elements of the IFNβ promoter. Cells were co-transfected with plasmids expressing the MGF genes indicated fused to a V5 tag, or Empty Vector or MC160 as controls. Cells were co-transfected with plasmids expressing renilla luciferase as internal control. Cells were stimulated with: **A.** TNFα and IL-1β, or transfected to exogenously express **B.** IKKα.flag, IKKβ.flag or **C.** p65-GFP. **DE.** Western blotting confirming the expression of exogenous stimulants, p65.GFP and IKKβ.flag in cells co-transfected with empty vector. **E.** Western blot confirming the expression of the V5-tagged MGFs, and MC160.HA. **DE.** Left hand values indicate the position of molecular weight markers run in parallel in kDa. β3-Tubulin was used as loading control. Western blotting is representative of experiments run in at least duplicate. **F.** Schematic highlighting (red stars) the level of the NFκB pathway where stimulation was induced to probe the pathway. Luciferase data is representative of three experiments. For statistical analysis two-way ANOVA Tukey's multiple comparison test was performed comparing stimulated Empty Vector to stimulated MGFs. **** = $p \leq 0.0001$, *** = $p \leq 0.001$, ** = $p \leq 0.01$, * = $p \leq 0.05$.

5.2.2 MGF360-12L & MGF505-1R Inhibit the NF κ B Pathway in a Dose Dependant Manner

Using the dual luciferase assay as described in 2.6, I investigated whether MGF360-12L and MGF505-1R were functioning in a dose dependant manner (Figure 5-3). HEK293T cells were transfected with reporter plasmids, constructs expressing p65-GFP as stimulus and increasing concentrations of MGF360-12L or MGF505-1R, from 50 ng to 350 ng. This revealed that both viral proteins inhibited activation of the luciferase reporter in a dose dependant manner. Interestingly MGF360-12L acted as an exceptionally strong inhibitor since transfection with only 50 ng of plasmid construct expressing the protein has a marked effect. This was not the case for MGF505-1R, where an inhibitory effect was not seen until transfection of 100 ng of the plasmid.

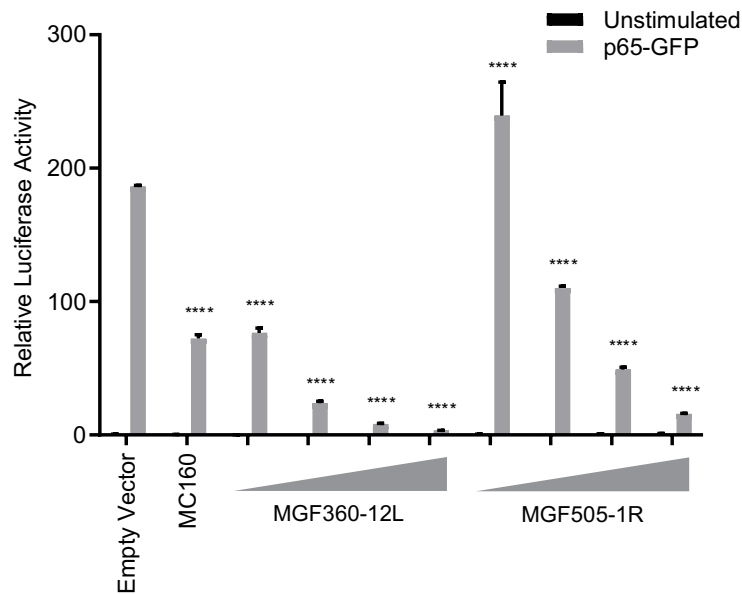
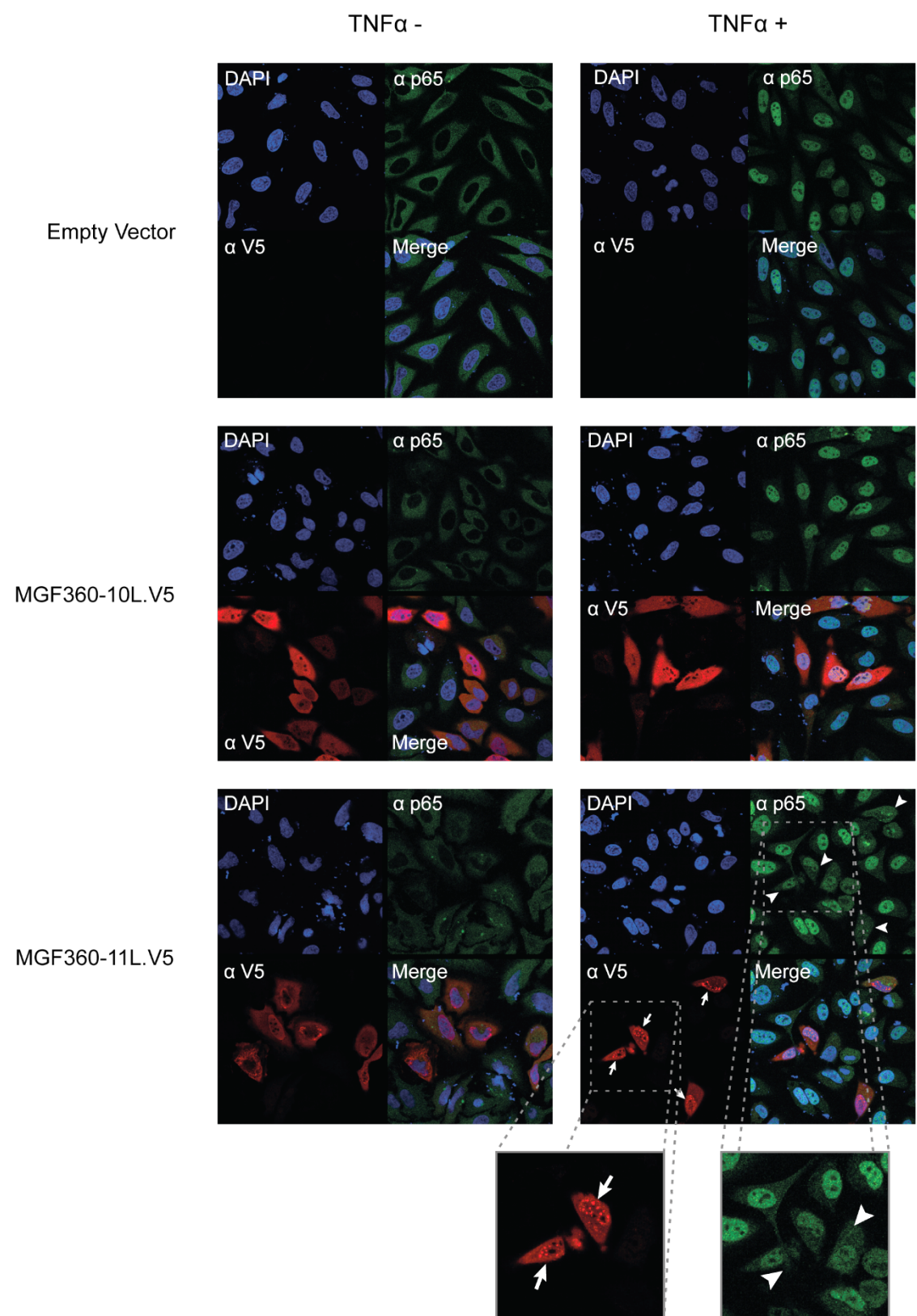
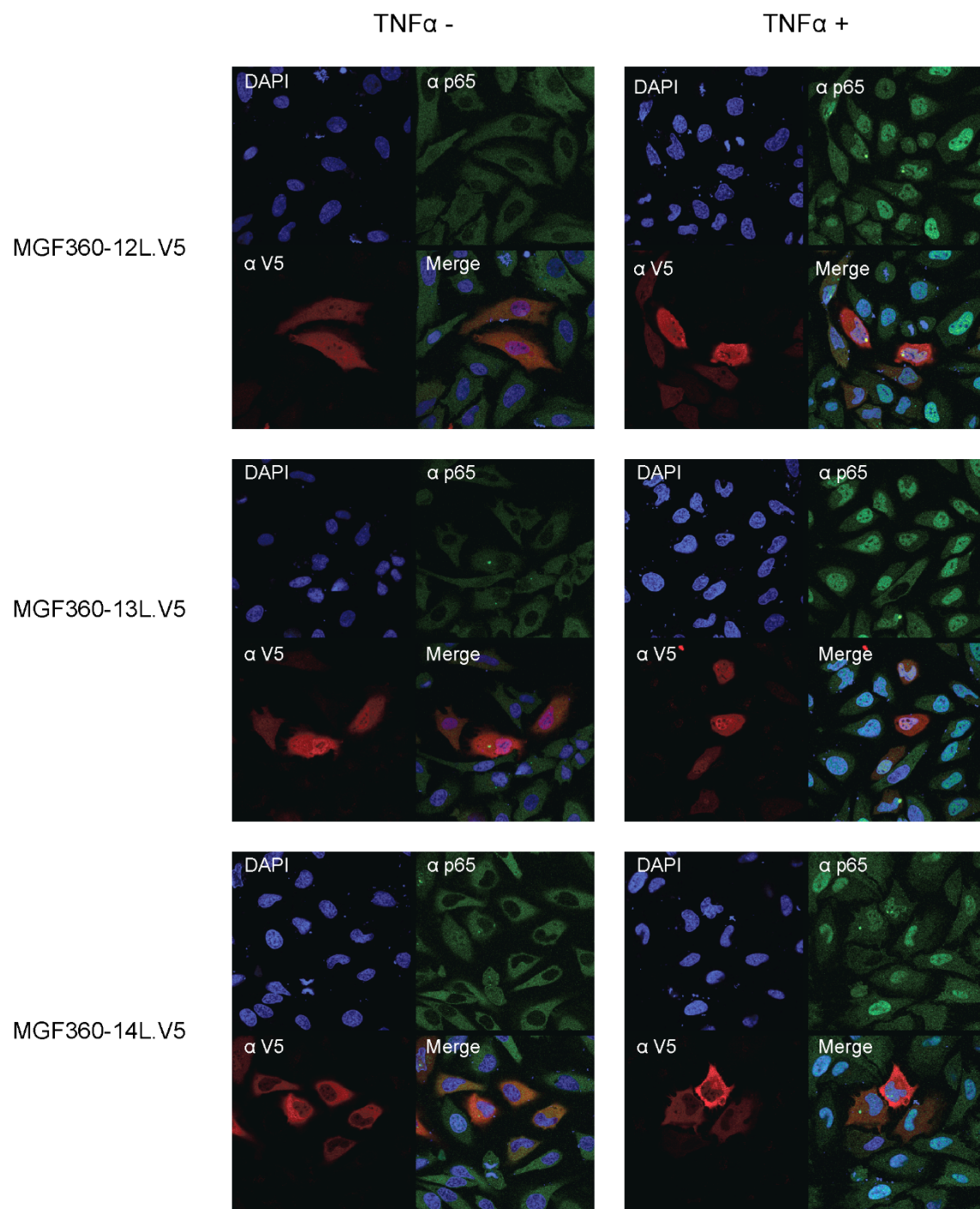


*Figure 5-3. Dose dependent inhibition of the NFκB pathway by MGF360-12L and MGF505-1R. Dual luciferase assays investigating inhibition of the firefly luciferase reporter gene under the control of the NFκB response elements in transfected HEK293T cells. MGF360-12L and MGF505-1R expressing plasmids were transfected at increasing concentrations (50 ng.well, 100 ng.well, 200 ng.well, 350 ng.well), or with controls of empty vector, or MC160. Data is representative of experiments performed in duplicate. For statistical analysis two-way ANOVA Tukey's multiple comparison test was performed comparing stimulated Empty Vector to stimulated MGFs. **** = $p \leq 0.0001$, *** = $p \leq 0.001$, ** = $p \leq 0.01$, * = $p \leq 0.05$.*

5.2.3 MGF360-11L & MGF505-2R Inhibited p65 Nuclear Translocation

As inhibition of NF κ B pathway was observed in cells transfected with a plasmid expressing p65.GFP suggesting inhibition at the transcription factor level or below, assessing whether the MGFs of interest inhibited nuclear translocation of p65 was an obvious line of investigation. Confocal immunofluorescence microscopy was used to investigate. HeLa cells were employed due to their relative ease of use. Cells were transfected with constructs expressing the MGFs fused to a V5-tag, or with empty vector pcDNA3.1 as negative control. Cells were subsequently either not stimulated or stimulated with TNF α for 20 ng.mL for 30 minutes. Cells were stained against V5-tag indicating MGFs (red – Adsorption 568nm) and endogenous p65 (green - Adsorption 488nm), and DAPI to highlight the nucleus (blue). Nuclear translocation of p65 was observed in stimulated control cells. Expression of V5-tagged MGF360-10L, 12L, 13, 14L, MGF505-1R and 3R did not appear to inhibit nuclear translocation of p65, however MGF360-11L and MGF505-2R showed weaker staining intensity in the nucleus, relative to adjacent cells negative for V5 staining (Figure 5-4 – See MGF360-11L and MGF505-2R arrowheads). Further assessment is required to confirm this effect, western blotting probing for p65 levels in nuclear extracts may offer more insight. Interestingly the MGFs presented with differing localisation patterns. MGF360-14L, and MGF505-2R were exclusively found in the cytoplasm suggesting their impact on the pathway is not nuclear, whereas the remaining MGFs were found in both in the nucleus and cytoplasm. Interestingly stimulation with TNF α resulted in punctate aggregation in the nucleus following transfection of plasmid expressing MGF360-11L (Figure 5-4 – See MGF360-11L arrows).





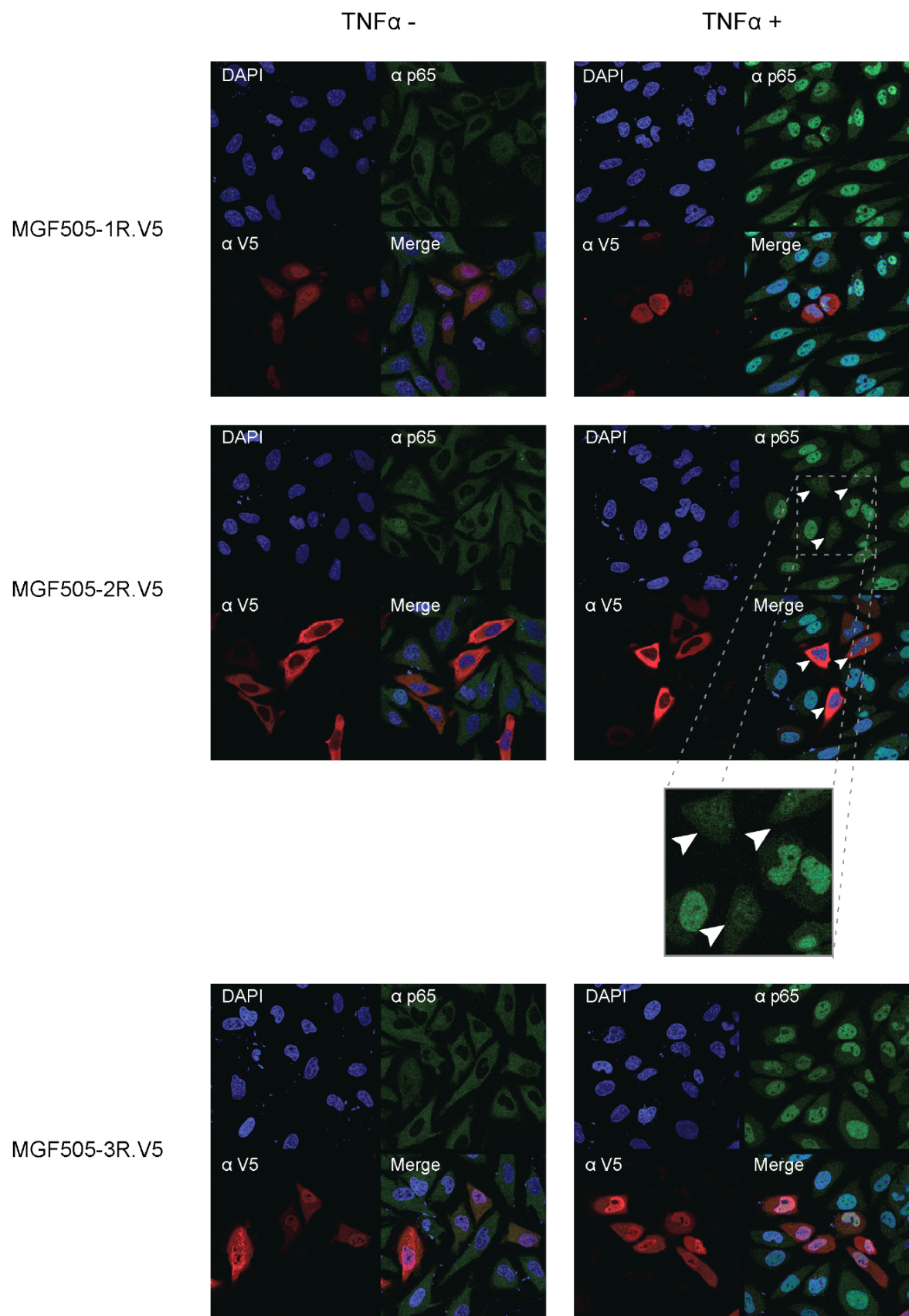


Figure 5-4. Impact of MGFs on nuclear translocation of p65 Confocal immunofluorescence showing Hela cells transfected with constructs expressing MGFs fused to V5-tag, stimulated with TNF α for 20 minutes, and probed for V5 (red), and endogenous p65 (green). Blue is DAPI nuclear staining. Nuclear translocation of p65 was observed in stimulated empty vector control cells. MGF360-11L, and MGF505-2R, impacted on nuclear p65 – see **arrowheads**. MGF360-11L.V5 shows a punctate aggregate of the viral protein in the nucleus on stimulation – see **arrows**.

5.2.4 MGF360-12L & MGF505-1R did not Impact the Expression of Selected Components of the NF κ B Pathway

Due to time and resource constraints I decided to focus on two MGF genes, MGF360-12L and MGF505-1R. Both of which showed interesting data earlier on in the investigation to follow up on, furthermore I demonstrated that these two genes are essential in the attenuation of virulent isolate Georgia07/1 (Chapter 8). Using western blotting I investigated the impact of MGF360-12L and MGF505-1R on key components of the NF κ B pathway (Figure 5-5AB). HEK293T cells were transfected with empty vector pcDNA3.1 as control, or constructs expressing MGF360-12L.V5 or MGF505-1R.V5. Cells were stimulated using TNF α (20 ng.mL) for a time course covering 0, 5, 15, 30 minutes. Both MGF360-12L.V5 and MGF505-1R.V5 could be clearly seen to be expressed, with strong bands at 38KDa and approximately 60KDa as expected for the predicted size (Figure 5-5AB – See V5). In empty vector pcDNA3.1, MGF360-12L.V5 and MGF505-1R.V5 transfected cells, an increase in phosphorylated p65 at residue S536 was observed at 5 minutes and peaked at 15 minutes post-stimulation, with a reduction at 30 minutes (Figure 5-5AB – See p-p65 (S536)). This coincided with the reduction of I κ B α levels across the time course (Figure 5-5AB – See I κ B α). These results suggest that the activation of I κ B α leading to its degradation and subsequent p65 activation was functional despite the presence of the viral proteins. Furthermore, no major difference was observed in IKK β levels. Together this indicates that MGF360-12L.V5 and MGF505-1R.V5 do not impair I κ B α degradation, nor p65 phosphorylation thus the activation of pathway remained functional until this point.

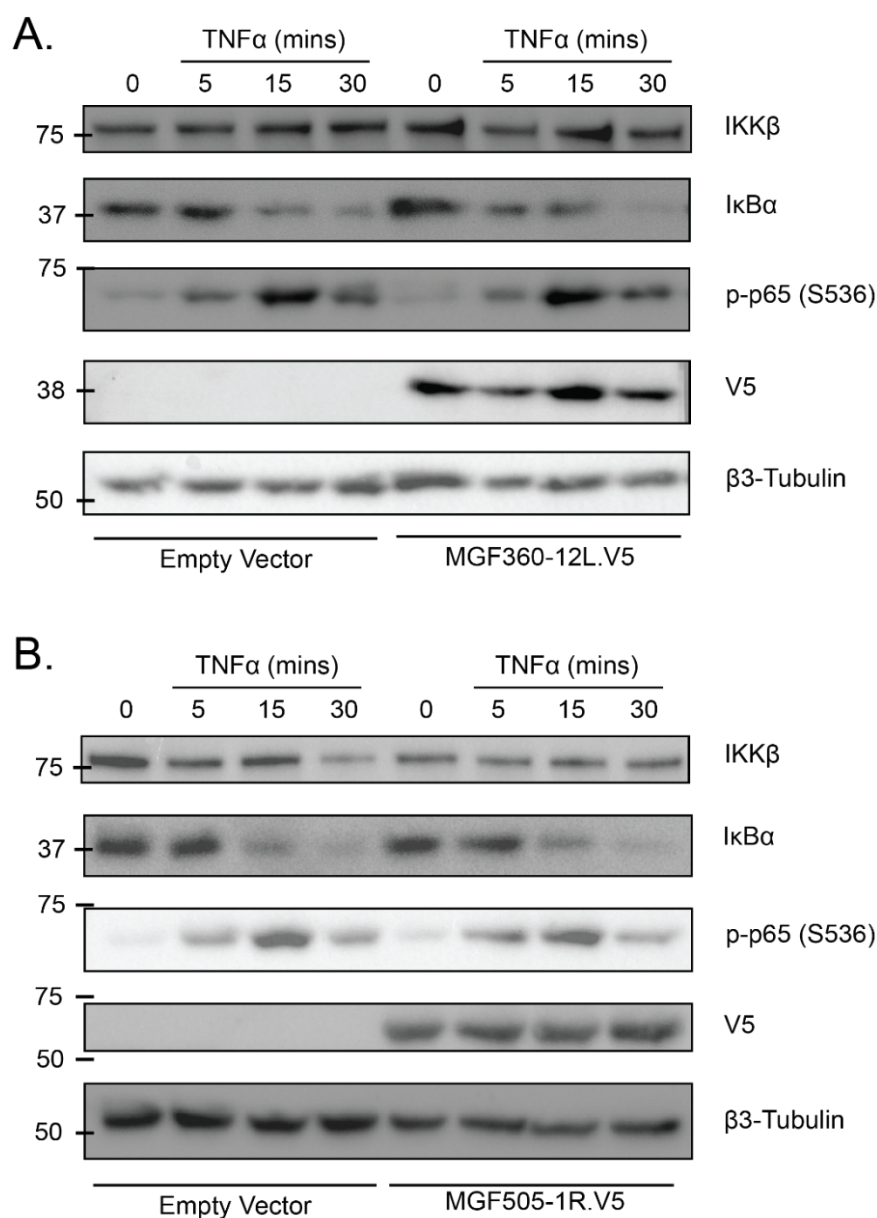


Figure 5-5. MGF360-12L and MGF505-1R do not impact on NF κ B pathway functionality HEK293T cells were transfected with pcDNA3.1 empty vector or **A.** MGF360-12L.V5. or **B.** MGF505-1R.V5. Cells were stimulated with TNF α for time period indicated in the top bar. Endogenous protein levels (see right-hand side) were assessed by western blotting following SDS-PAGE separation. Left-hand values indicate molecular weight in kDa. β 3-Tubulin was used as endogenous loading control. Westerns blots are representative of experiments performed in duplicate.

5.2.5 Impact of MGF360-12L's Putative Novel TRAF6 Binding Site on Inhibition of the NF κ B Pathway

Previous work (Chapter 3) demonstrated that MGF360-12L possesses a putative novel TRAF6 binding motif, and moreover was shown to be a positive interactor with this E3 Ub ligase protein using yeast-2-hybrid assay (See 4.2.3). Here I used CoIP to further investigate interactions between the two proteins. Using site-directed mutagenesis I generated a MGF360-12L.V5 mutant replacing three key residues of the novel TRAF6 binding motif with alanine with the aim of disrupting the motif (MGF360-12L.V5.TRAF6ala) (Figure 5-6A).

HEK293T cells were transfected with empty vector pcDNA3.1, or pcDNA3.1 expressing MGF360-12L.V5 or mutant variant MGF360-12L.V5.TRAF6ala. Cells were co-transfected with and pRK5.flag.TRAF6, or equal amount of pcDNA3.1 empty vector. Mouse anti-V5 antibodies covalently coupled to EpoxyM270 Dynabeads were used to pull down proteins. Western blotting of input lysate confirmed the expression of flag.TRAF6 and MGF360-12L.V5 and mutant MGF360-12L.V5.TRAF6ala. Interestingly I observed a drop off in TRAF6 level in the presence of both MGF360-12L.V5 and MGF360-12L.V5.TRAF6ala relative to empty vector (Figure 5-6B - See Input Flag). Western blotting of the pull-down confirmed the presence of MGF360-12L.V5 and MGF360-12L.V5.TRAF6ala therefore indicating that the pull-down had been successful in isolating the V5 expressing proteins. However, I was unable to identify flag.TRAF6 in the pull-down (Figure 5-6B). Further work is required to assess this potential interaction. Interestingly, using luciferase assay, I demonstrated that the mutant MGF360-12L.V5.TRAF6ala partially rescued inhibition when the NF κ B pathway was

stimulated with TNF α (Figure5-6C), or by exogenous expression of TRAF6 (Figure5-6D). The results hint towards a dual function of MGF360-12L with the inhibition.

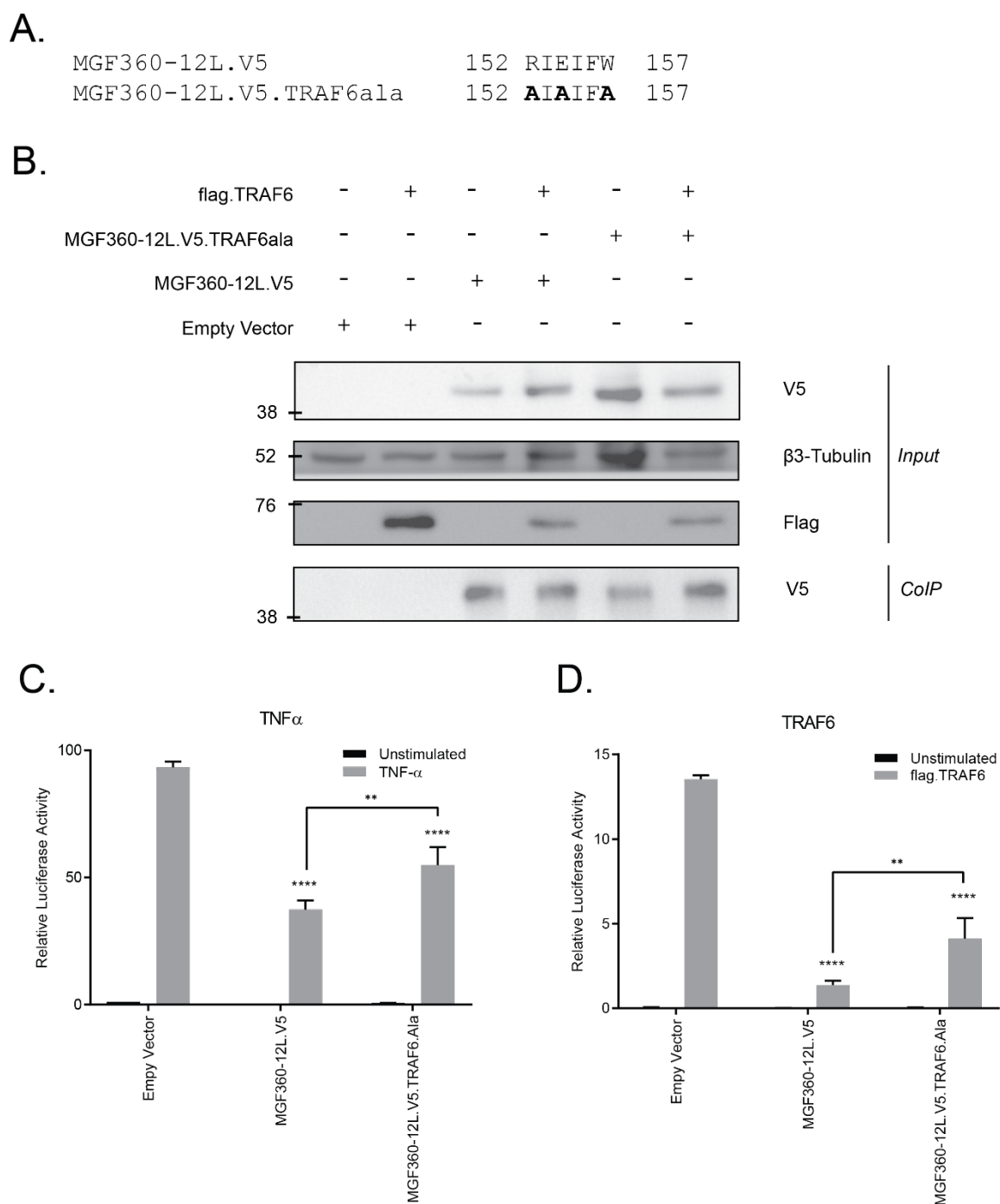


Figure 5-6. Impact of mutation of the putative novel TRAF6 binding motif in MGF360-12L on inhibition of the NFκB pathway **A.** Sequences showing wild-type MGF360-12L and mutations introduced in aim of disrupting the identified putative novel TRAF6 binding motif. Mutated residues are in bold. **B.** CoIP investigating interactions between MGF360-12L and TRAF6. HEK293T cells were transfected with empty vector or MGF360-12L.V5 or mutant variant MGF360-12L.V5.TRAF6ala.

Cells were co-transfected with plasmid expressing flag.TRAF6. Cells were lysed and sample taken as input or incubated with magnetic beads bound to anti-V5 antibodies as CoIP. Input and CoIP were analysed by SDS-PAGE probing with anti-V5 antibodies, or anti-flag antibodies. CoIP of flag.TRAF6 was unsuccessful (data not shown). Molecular weight protein ladders were run in parallel and their position in kDa are noted on the left-hand side. β 3-Tubulin was used as endogenous loading control. **CD.** HEK293T cells transfected with firefly luciferase reporter under the control of NF κ B response elements, and co-transfected with plasmids expressing pcDNA3.1 empty vector, MGF360-12L, or MGF360-12L.V5.TRAF6 Δ ala. Cells were stimulated with TNF α (C) or co-transfected to exogenously express flag.TRAF6 (D). Luciferase Data is representative of duplicate experiments. For statistical analysis two-way ANOVA Tukey's multiple comparison test was performed comparing stimulated Empty Vector to stimulated MGFs. Bar indicates comparison between MGF360-12L.V5 and MGF360-12L.V5.TRAF6 Δ ala. **** = $p \leq 0.0001$, *** = $p \leq 0.001$, ** = $p \leq 0.01$, * = $p \leq 0.05$.

5.2.6 MGF360-12L did not Co-Immunoprecipitate with Endogenous TRAF6 or p65

As the data here indicated that MGF360-12L may be impacting upon NF κ B and/or TRAF6, assessing potential interaction with the endogenous proteins was an obvious route of investigation. CoIP was performed to probe for an interaction between MGF360-12L with TRAF6, and p65. HEK293T cells were transfected with empty vector pcDNA3.1 as control, or MGF360-12L.V5. Cells were incubated in DSP crosslinking agent. Using magnetic beads coupled to anti-V5 antibodies MGF360-12L.V5 was successfully immunoprecipitated (Figure 5-7 Lane 4). However, probing the immunoprecipitate for interacting partners was unsuccessful (Figure 5-7 Lane 4 - TRAF6 and p65). However, it is of note that in contrast to exogenously expressed TRAF6 (Figure 5-6B), here MGF360-12L did not impact upon the level of endogenous TRAF6 (Figure 5-7 Lane 2).

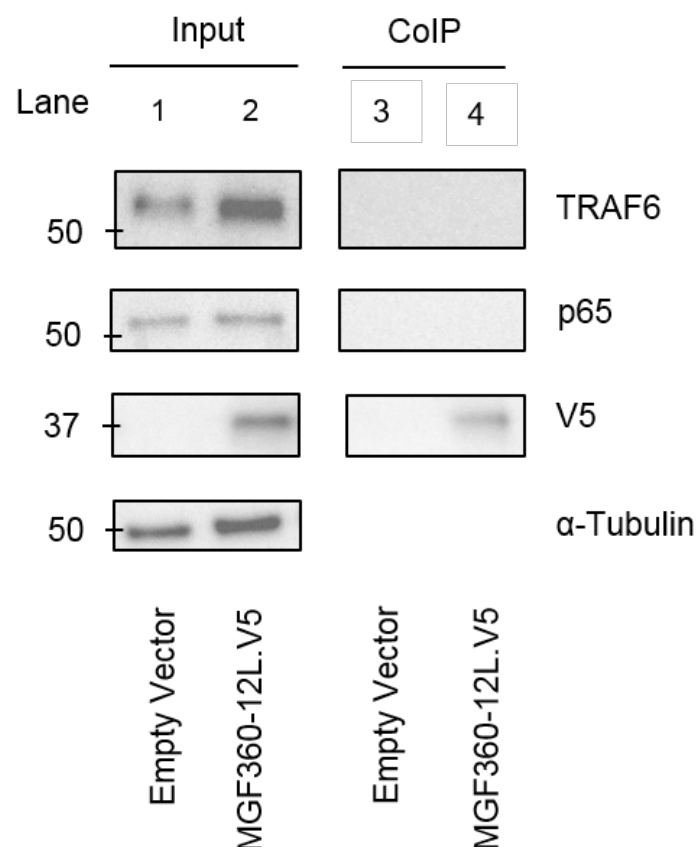


Figure 5-7. MGF360-12L did not co-immunoprecipitate with endogenous p65 or TRAF6 HEK293T cells were transfected with empty vector pcDNA3.1 or MGF360-12L.V5. Cells were incubated in DSP crosslinking agent and lysate sample was taken as **Input**, or incubated in magnetic bead covalently linked to anti-V5 antibodies as **CoIP**. Input and CoIP were probed for V5, and TRAF6 or p65, via western blotting following SDS-PAGE separation. Left hand values indicate the position of molecular weight markers run in parallel in kDa. α-Tubulin was used as endogenous loading control. Assay was performed in concurrence with CoIP in Figure 6-5, Figure 7-3 and Figure 7-8. Therefore α-Tubulin endogenous control, and V5 probing is the same.

5.2.7 MGF505-1R did not Co-Immunoprecipitate with Endogenous p65

CoIP was performed to assess a potential interaction between MGF505-1R and endogenous p65 (Figure 5-8). HEK293T cells were transfected with plasmid expressing the viral protein MGF505-1R.V5 or empty vector pcDNA3.1. Cells were treated with DSP crosslinker prior to lysis. Sample of lysate was taken as input (Figure 5-8 Lane 1 and 2). Remaining lysate was incubated in magnetic beads coupled to V5 antibodies and probed by western blotting (Figure 5-8 Lane 3 and 4). Probing with V5 antibodies revealed a successful pull-down MGF505-1R.V5 (Figure 5-8 Lane 4). However despite input showing presence of endogenous p65 (Figure 5-8 Lane 1 and 2), no banding corresponding to p65 could be observed in the CoIP (Figure 5-8 Lane 4), indicating that MGF505-1R.V5 and p65 were not interacting.

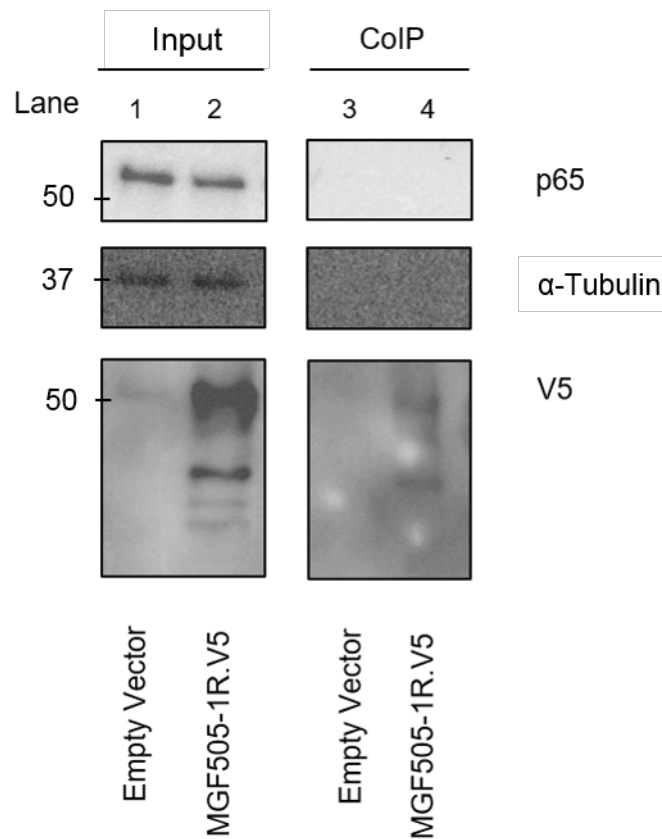


Figure 5-8 MGF505-1R did not co-immunoprecipitate with endogenous p65
*HEK293T cells were transfected with pcDNA3.1 empty vector, or MGF505-1R.V5. Cells were incubated in DSP crosslinking agent prior to lysis. Sample of lysate was taken as **Input**. Remaining lysate was incubated in magnetic beads coupled to anti-V5 antibodies for **CoIP**. Samples were probed for V5, α-Tubulin endogenous loading control, and p65 by western blotting following SDS-PAGE separation. Left hand values indicate the position of molecular weight markers run in parallel in kDa. Experiment was performed in concurrence with CoIP in Figure 7-4A and Figure 7-7B. Therefore, α-Tubulin endogenous control and V5 probing is the same.*

5.2.8 MGF360-12L Inhibits p65 Transcriptional Activity in a Luciferase Assay Under the Control of the GAL4 promoter

Here I showed that the MGFs were capable of inhibiting the NFκB pathway at the transcription factor level or below (Figure 5-2), but did not observe a reduction in the p65 nuclear translocation (except MGF360-11L, and MGF505-2R) (Figure 5-4) nor an impact the functional activity of the pathway with MGF360-12L and MGF505-1R (Figure 5-5). I therefore proposed that the inhibition may be occurring by directly inhibiting NFκB transcriptional activity, by interfering with cofactor machinery or blocking transcription factor binding to promoter DNA. To investigate these possibilities HEK293T cells were transfected with a construct expressing firefly luciferase under the control of GAL4 promoter, and a Renilla construct as background control. Cells were stimulated by the exogenous expression of Gal4 DBD (including nuclear localisation signal) fused to p65 known as Gal4DBD-p65. Therefore, expression of Gal4DBD-p65 would induce expression of luciferase in this system as the Gal4DBD domain would bring the fusion protein into proximity to the promoter, whilst p65 provides the required TAD. Gal4-147 included the DBD of Gal4 alone, and thus would not stimulate luciferase expression due to the absence of the transcription activation domain. Using this system, I did not observe inhibition of stimulation in cells expressing MC160, MGF360-10L, 11L, 13L, 14L, MGF505-1R, 2R, and 3R (Figure 5-9), and therefore can conclude that these proteins are not inhibiting the p65 transactivation activity. Thus, the inhibition by these proteins seen in Figure 5-2C may be interfering with NFκB DNA binding capacity. As expected, the overexpression of Gal4-147 also resulted in inhibition likely by competing with

the fusion protein for binding to the GAL4 promoter. Interestingly, significant inhibition was observed in cells expressing MGF360-12L (Figure 5-9). This may indicate that MGF360-12L is inhibiting the transcriptional activity of p65 in this system. MGF360-12L's inhibition of the NF κ B pathway therefore may be explained by interference with transcriptional activity domain of the transcription factor, or indirectly via cofactors (Figure 5-10).

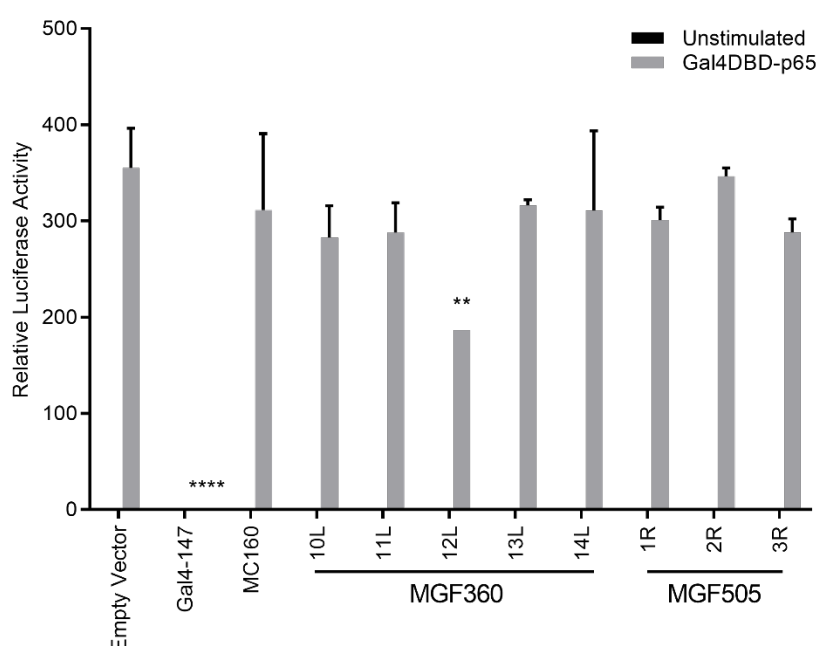


Figure 5-9. Analysis of MGFs on the transcription activity of Gal4DBD-p65 HEK293T cells were transfected with constructs expressing Gal4-luciferase reporter gene and renilla internal control. Cells were co-transfected with MGFs, or Gal4-147 or MC160 controls. Stimulated cells were co-transfected with Gal4DBD-p65, or in unstimulated cells co-transfected with equal amounts of pcDNA3.1 empty vector. Data is representative of three experiments. For statistical analysis, two-way ANOVA Tukey's multiple comparison test was performed comparing stimulated Empty Vector to stimulated MGFs. **** = $p \leq 0.0001$, *** = $p \leq 0.001$, ** = $p \leq 0.01$, * = $p \leq 0.05$.

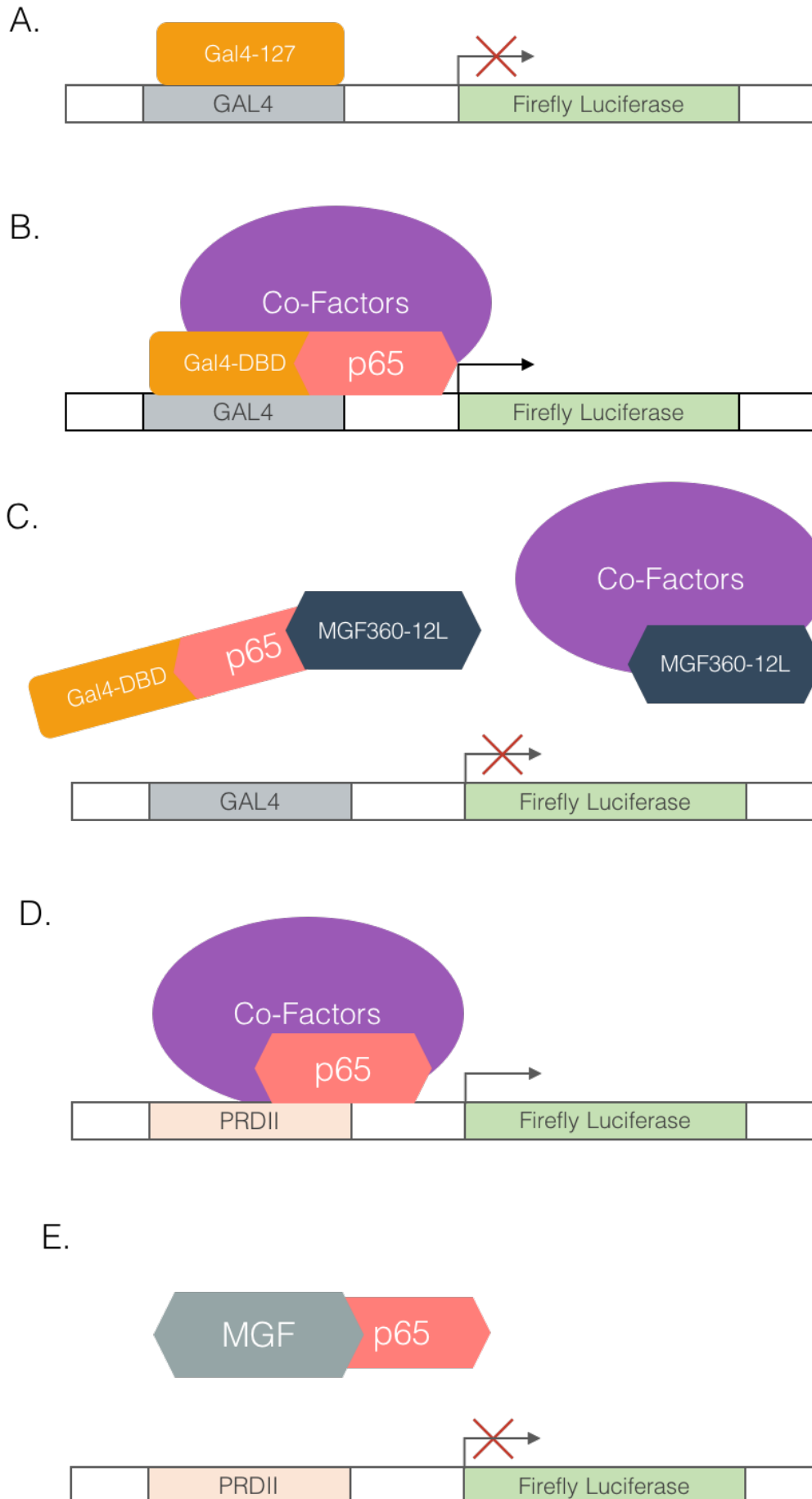


Figure 5-10. Schematic giving overview and explanation of data observed from luciferase assays. **A.** Gal4 luciferase system in the presence of Gal4-14 , note the lack of firefly luciferase expression. **B.** Stimulation of the Gal4-Luciferase system in the presence of Gal4DBD-p65. **C.** MGF360-12L inhibiting Gal4-p65 assay potentially via blocking transcriptional activity or cofactor **D.** Firefly luciferase under the control of PRDII (NFκB binding region of IFN promoter) stimulated by p65 activation. **E.** Potential MGF (excluding MGF360-12L) inhibition mechanisms of p65 stimulated firefly luciferase under the control of NFκB regulatory elements.

5.3 Discussion

Here I demonstrate the ability of the MGFs of interest to inhibit the NFκB pathway and begin to make inroads in unravelling the inhibitors' mechanism of action (See Table 5-1). The NFκB response can be divided into two pathways, the canonical and the alternative. Here I refer to the canonical pathway. The NFκB pathway is a key signalling cascade in the development of an immune response to pathogens (Li and Verma 2002, Pfeffer 2011, Liu, Zhang *et al.* 2017). Upon triggering of PRRs including TLRs, RLRs, DNA sensors by PAMPs, or activation of TNFR, and IL1R receptors by their respective cytokines, activation of the NFκB pathway is induced (See 1.1.5.1 and 1.1.5.2). Activation of these signalling pathways leads to activation of the IKK complex and ultimately the NFκB transcription factor (See 1.1.4.2).

MGF	Inhibition of NFκB dependent luciferase - location	Cellular localization	Inhibition of p65 Nuclear translocation?	Inhibition of the gal4 luciferase?	Other
MGF360-10L	At p65 level or below	Cytoplasmic & Nuclear	No	No	
MGF360-11L		Cytoplasmic, but stimulation showed nuclear localization with apparent aggregates	Yes	No	
MGF360-12L		Cytoplasmic & Nuclear	No	Yes	Mutation of TRAF6 binding motif alleviated inhibition. No Impact on NFκB phosphorylation or IκBα degradation observed. No interaction with p65 or TRAF6 observed.
MGF360-13L		Cytoplasmic & Nuclear	No	No	
MGF360-14L		Cytoplasmic	No	No	
MGF505-1R		Cytoplasmic & Nuclear	No	No	No Impact on NFκB phosphorylation or IκBα degradation observed. No interaction with p65 observed.
MGF505-2R		Cytoplasmic	Yes	No	
MGF505-3R		Weakly cytoplasmic & Strong nuclear	No	No	

Table 5-1. Overview of the results of the impact of the MGFs on the NFκB signalling pathway

By stimulating the NF κ B signalling pathway by using TNF α , IL-1 β , or exogenous expression of key downstream components IKK α , IKK β and p65 I established that MGF360-10L, 11L, 12L, 13L, 14L and MGF505-1R, 2R, 3R were inhibitors of the pathway. Importantly, by systematically probing the pathway I identified that the location of inhibition was at the transcription factor level or below (Figure 5-2). As activation of NF κ B leads to its nuclear translocation, attempting to ascertain whether the MGFs were inhibiting the pathway by blocking the transcription factor ability to reach its nuclear target was of a clear interest. Using confocal microscopy, I observed that the MGFs did not have an obvious impact on p65 nuclear translocation with the exception of MGF360-11L and MGF505-2R (Figure 5-4). Although most MGFs were observed in both the nucleus and cytoplasm, curiously MGF360-14L and MGF505-2R were only found in the cytoplasm. Furthermore MGF360-11L showed an altered localisation phenotype on stimulation. These differences therefore may indicate that the demonstrated inhibition of the pathway by these MGFs is likely occurring via different mechanisms. MGF505-2R's cytoplasmic localisation and reduction in nuclear p65 observed on stimulation may be explained by the viral protein sequestering the transcription factor in the cytoplasm, further analysis is needed to confirm this, for example by western blotting of nuclear fraction. Interestingly MGF360-11L was found in the cytoplasm prior to treatment and appeared to migrate to the nucleus with stimulation, whether this was in coordination with NF κ B remains to be seen.

The lack of impact on the activation of p65 by MGF360-12L and MGF505-1R was confirmed by western blotting showing neither proteins prevented the induction of phosphorylated p65 (Figure 5-5). The presence of increasing levels of phosphorylated and thus active p65, and rapid I κ B α degradation after only 15 minutes of stimulation as anticipated, indicate that the pathway is functional in the presence of MGF360-12L, and MGF505-1R. Furthermore, neither protein was successfully co-immunoprecipitated with p65 (Figure 5-7 and Figure 5-8). However, the caveat remains that inefficient transfection may result in insufficient quantities of viral protein in both western blots and coimmunoprecipitations, which thus may be masking any potential impact of the MGFs on the cellular proteins. Determining transfection efficacy or repeating the assays after the isolation of transfected cells would address this.

As the impact on the pathway was occurring at the transcription factor level or below, assessment on transcriptional activity was performed. I employed an alternate luciferase assay to achieve this. By controlling firefly luciferase with a GAL4 promoter I was able to stimulate luciferase expression upon transfection of a construct expressing Gal4DBD-p65 fusion protein. This fusion protein could bind to the GAL4 promoter region and utilise the TAD of p65 to induce the expression of luciferase. Therefore, if no inhibition in the Gal4 system was observed, this would indicate that the inhibition of the luciferase assay under the control of PRDII region, as in Figure 5-2, would be due to impeding the DNA binding activity or domain of p65 rather than impacting on its transcriptional activity. Whereas if inhibition were observed in this Gal4 luciferase system it

would indicate that this was via targeting p65's transcriptional activity, or indirectly by impeding transcriptional cofactors (Figure 5-10). Interestingly I only observed inhibition of the Gal4 luciferase system with MGF360-12L. I therefore proposed that the MGFs, with the exception of MGF360-12L, do not directly inhibit NFκB transcriptional activity. Overall, the data here would strongly suggest that despite inhibition at approximately the same level within the pathway the MGFs appear to function with a variety of mechanisms.

There are numerous methods that the MGFs could be deploying to inhibit NFκB activity. Western blotting showing consistent levels of endogenous proteins, including loading control β-Tubulin, with MGF360-12L and MGF505-1R would indicate that the inhibition for these two genes at least is not broad spectrum translational inhibition. NFκB induced gene expression by RNAPII is reliant on the presence of cofactors including CBP, and the highly related p300 (Gerritsen, Williams *et al.* 1997). CBP/p300 kinase-inducible domain interacting domain (KIX) forms protein-protein contact with rel homology domain of active p65 bound to the promoter region, enabling an architecture to facilitate transcription by TFIIB, RNAPII enhancer complex (Zhong, Voll *et al.* 1998). Interestingly, Mukherjee, Behar *et al.* (2013) demonstrated that CBP is constitutively expressed and preloaded to NFκB promoter regions, and De Bosscher, Vanden Berghe *et al.* (2000) suggested that CBP is required for Gal4DBD-p65 Gal4 driven gene expression. Thus, the lack of inhibition seen in the Gal4 luciferase system (Figure 5-9) signifies that MGFs, bar MGF360-12L, are unlikely to be disrupting this specific transcriptional machinery. Therefore, the inhibition of the NFκB pathway

(Figure 5-2) but the lack of Gal4DBD-p65 inhibition may be explained by disruption of p65 binding to promoter DNA. Assessing whether the MGFs were blocking NFκB DNA binding by electrophoretic mobility shift assay (EMSA) would be of obvious interest. MGF360-12L however may be interfering with transcriptional mechanics as it also demonstrated inhibition of Gal4DBD-p65. This could occur in a variety of manners, including directly blocking the p65 TAD, or by interfering with cofactor activity.

Due to the previous finding that MGF360-12L contains a novel TRAF6 binding motif (See 3.2.5) but also yielded a positive interaction with the cellular protein in the yeast-2-hybrid assay (See 4.2.3), I therefore investigated whether this viral protein had an impact on TRAF6. TRAF6 is a RING finger protein with E3 Ub ligase activity and has been shown to be crucial in the NFκB activation pathway (Shi and Sun 2018). By stimulating cells with TNFα or exogenous TRAF6 expression I was able to see a partial rescue in inhibition with MGF360-12L containing a destructive mutation in the novel TRAF6 binding motif (MGF360-12L.V5.TRAF6ala) (Figure5-6). Moreover, to confirm the yeast-2-hybrid results and the functionality of the novel TRAF6 binding motif, I attempted to pull-down exogenously expressed flag.TRAF6 via CoIP. Although the immunoprecipitation was successful as I could detect both MGF360-12L.V5 and MGF360-12L.V5.TRAF6ala in the pull-down, I was however unable to detect flag.TRAF6. The inability to successfully co-immunoprecipitate flag.TRAF6 could potentially be explained if the viral protein was utilising putative VHL/SOCS E3 CRL ligase machinery leading to the proteasomal destruction of the bound cellular protein.

Interestingly the observation of a reduction in flag.TRAF6 in the presence of both wild-type and mutant MGF360-12L, may hint towards this (Figure 5-6B), although this was not the case of endogenous protein (Figure 5-7), thus whether MGF360-12L is targeting TRAF6 for proteasomal destruction remains to be seen. Curiously, the introduction of the mutation did not alter the level of flag.TRAF6 relative to the wild-type protein, potentially indicating that the mutation did not have an impact on function. However, this is at odds with the luciferase data showing a partial rescue of inhibition (Figure 5-6C). It cannot be ruled out that the introduction of the mutation disrupted the architecture of the protein therefore reducing its inhibitory capacity. CoIP using a truncated MGF360-12L lacking the potential VHL/SOCS-Box could help reveal the binding target(s), if these are indeed being targeted for proteasomal degradation.

The NFκB pathway clearly plays a crucial role in the development of an adequate immune response, it is therefore unsurprising that the MGFs inhibit the NFκB pathway at the transcriptional factor level or below. I have also proposed that MGF360-12L acts with dual function, inhibition of TRAF6 and the transcriptional machinery to inhibit the NFκB pathway. Overall, the variation in results between the MGFs of interest clearly shows that they are acting at a similar level within the pathway but likely with differing mechanisms of action.

6 INHIBITION OF THE IRF3 SIGNALLING PATHWAY BY MGF360 & MGF505 PROTEINS

6.1 Introduction

As previously demonstrated elsewhere the combined absence of MGF360-10L, 11L, 12L, 13L, 14L and MGF505-1R, 2R, 3R are believed to be responsible for the inhibition of the innate immune system (See 1.2.4) (Afonso, Piccone *et al.* 2004, Golding, Goatley *et al.* 2016, Reis, Abrams *et al.* 2016). I have shown here the impact of these individual proteins on the NFκB pathway (See Chapter 5), and thus it is a logical step to investigate any effect on the IRF3 driven IFN response. Here I show that expression of individual MGF360-10L, 11L, 12L, 13L, 14L and MGF505-1R, 2R and 3R genes inhibit the IRF3 pathway at the transcription factor level or below.

A prompt response to invading pathogens is key to the development of immunity. This response is coordinated by numerous signalling cascades including the IRF pathway, once activated this pathway controls the induction of type-I IFN expression (See 1.1.4.1, Figure 1-1, and Figure 6-1). Here I will focus upon IRF3, which has been shown to be crucial in the development of an IFN response to viral pathogens (Sato, Suemori *et al.* 2000, Honda, Takaoka *et al.* 2006). Induction of the IRF3 pathway is initiated by the stimulation of PRRs by their respective stimulant PAMPs (Honda and Taniguchi 2006). In the context of viral pathogens this can occur in two broadly defined areas, transmembrane PRRs (See 1.1.5.1)

and cytosolic PRRs (See 1.1.5.2). Activation these PRRs leads to activation of downstream kinase TBK1 that is responsible for the phosphorylation and activation of IRF3 (See 1.1.4.1).

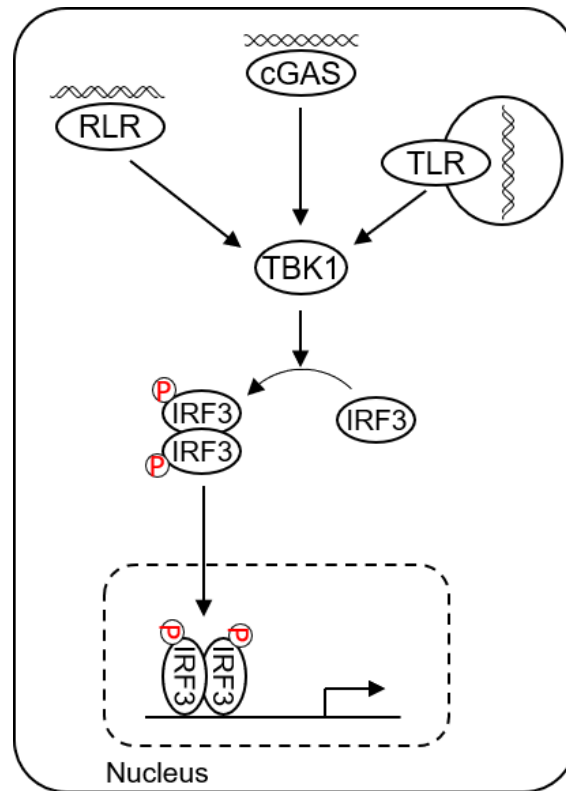


Figure 6-1. Schematic showing simple overview of activation of the IRF3 pathway

The activity of IRF3 relies on IKK kinases TBK1 and IKK ϵ to induce the phosphorylation and activation of the transcription factor (Fitzgerald, McWhirter *et al.* 2003). Although both kinases are implicated in the activation of IRF3, most research concentrates on TBK1 as it considered the principal contributor (Honda and Taniguchi 2006). I will therefore do the same. Stimulation of both the transmembrane and cytosolic PRRs involved in the IRF3 pathway strikingly all converge at TBK1 in order to activate IRF3 (Liu, Cai *et al.* 2015). RLR and TLR3

utilise TRAF3 to recruit the kinase along with scaffold proteins, of which three have been described: TANK, NAP1 and SINTBAD. This is in a similar fashion to IKK α/β requiring master regulator IKK γ to form the IKK complex, although less well described. All three scaffold proteins contain a TBK1 binding domain leading to complex formation with the kinase, moreover they are reported to be essential for virally driven TLR and RLR signalling cascades leading to TBK1 dependant IRF3 phosphoactivation (Sasai, Shingai *et al.* 2006, Ryzhakov and Randow 2007). However, their involvement in cGAS-STING-TBK1 pathway remains elusive where STING alone appears to be the driving force behind TBK1 activation (Tanaka and Chen 2012). Interestingly there specificity of the adaptor proteins associated with TBK1 depending on the origin of the pathway stimulation (Chau, Gioia *et al.* 2008). This is thought to be mediated by NAP1/TANK/SINTBAD inducing differing subcellular localisation of TBK1 and therefore driving interactions with differing signalling pathways (Goncalves, Bürckstümmer *et al.* 2011, Helgason, Phung *et al.* 2013). However, all three scaffold proteins are thought to be required for cytosolic PRR induced IRF3 activation (Chau, Gioia *et al.* 2008). TBK1 activates IRF3 by phosphorylating the transcription factor at two regions termed site 1, and site 2. Phosphorylation at serine residues at site 1 (S386) and site 2 (S396) are both required for full activation (Lin, Heylbroeck *et al.* 1998, Mori, Yoneyama *et al.* 2004, Panne, McWhirter *et al.* 2007, Chen, Srinath *et al.* 2008). Initial phosphorylation at S396 allows interaction with CBP cofactor, in the nucleus, and further phosphorylation of S386 induces the dimerisation necessary for activation (Panne, McWhirter *et al.* 2007). Active IRF3 dimer translocates to the nucleus, where it forms a complex with cofactors CBP/p300 (Lin, Heylbroeck *et al.* 1998, Lin, Génin *et al.* 2000, Panne, McWhirter *et al.* 2007). IRF3 binds

conserved promoter sites via the N-terminal DBD along with cofactors and components of the enhanceosome to induce the expression of type-I IFN genes (Thanos and Maniatis 1995, Taniguchi, Ogasawara *et al.* 2001, Panne, Maniatis *et al.* 2004, Honda and Taniguchi 2006). IFN itself will act in autocrine and paracrine fashion, leading to further expression changes of over 300 ISGs that are responsible for the creation of an “anti-viral environment” in order to gain control over the pathogen (See 1.1.6) (Schneider, Chevillotte *et al.* 2014).

The IRF3 signalling pathway is clearly vital in the development of an innate immune response to viral infection. It is therefore understandable why it would be advantageous for viruses to inhibit the pathway, thus preserving a more favourable cellular environment. In fact, inhibition of the pathway has been reported numerous times, with viruses targeting a wide range of components from each of the PRR pathways (See 1.1.7.1) (Randall and Goodbourn 2008). The evidence to suggest that ASFV is inhibiting the IFN β response provides insight that the virus is no exception (see 1.2.4 and 1.2.5). Due to their impact on the IFN pathway I therefore proposed that the MGF genes of interest (MGF360-10L, 11L, 12L, 13L, 14L, and MGF505-1R, 2R and 3R) may be inhibiting the IRF3 pathway to achieve this. Here I used luciferase assays coupled with confocal microscopy and western blotting to demonstrate inhibition of the IRF3 pathway is occurring at the transcription factor level or below, in a manner similar to that of their inhibition of the NF κ B pathway.

6.2 Results

6.2.1 MGF360 & MGF505 Inhibited the IRF3 Pathway at the Transcription Factor Level or Below

In a similar manner to the luciferase assay used to probe the NF κ B pathway (See 5.2.2), I used a luciferase firefly reporter construct under the control of the IRF3 response element of the IFN β gene as described in section 2.6. Upon stimulation and activation of IRF3, this would induce expression of firefly luciferase. Therefore, any reduction in luciferase expression can be considered inhibition of the pathway. By stimulating the pathway at differing levels, I was able to systematically probe the location of any inhibition within the cascade. Cantell strain of Sendai virus, and exogenous expression of TBK1.flag, or IRF3-5D a constitutively active variant of IRF3 were used to probe the pathway. HEK293T cells were transfected with constructs expressing the reporter gene, genes of interest, and in the case of exogenously stimulated cells plasmids coding for stimulants, TBK1.flag or IRF3-5D. Unstimulated cells were transfected with equal quantity of empty vector pcDNA3.1 to ensure the amount of DNA transfected was consistent with stimulated cells. Sendai virus was used to stimulate the IRF3 pathway due to its previously reported RLR activation (Kato, Takeuchi *et al.* 2006, Martínez-Sobrido, Zúñiga *et al.* 2006, Rehwinkel, Tan *et al.* 2010, Mathers, Schafer *et al.* 2014). A construct expressing classical swine fever virus IRF3 inhibitor NPro protein was used as a positive control (Seago, Hilton *et al.* 2007, Gottipati, Holthauzen *et al.* 2016).

Using this assay I saw a significant decrease in relative firefly luciferase expression in the presence of MGFs and positive control when stimulated with Sendai virus (Figure 6-2A), TBK1.flag (Figure 6-2B) and IRF3-5D (Figure 6-2C), indicating inhibition is occurring at the transcription factor level or below. Interestingly this is similar to observations from inhibition in the NF κ B pathway, potentially hinting towards a common mechanism of inhibition.

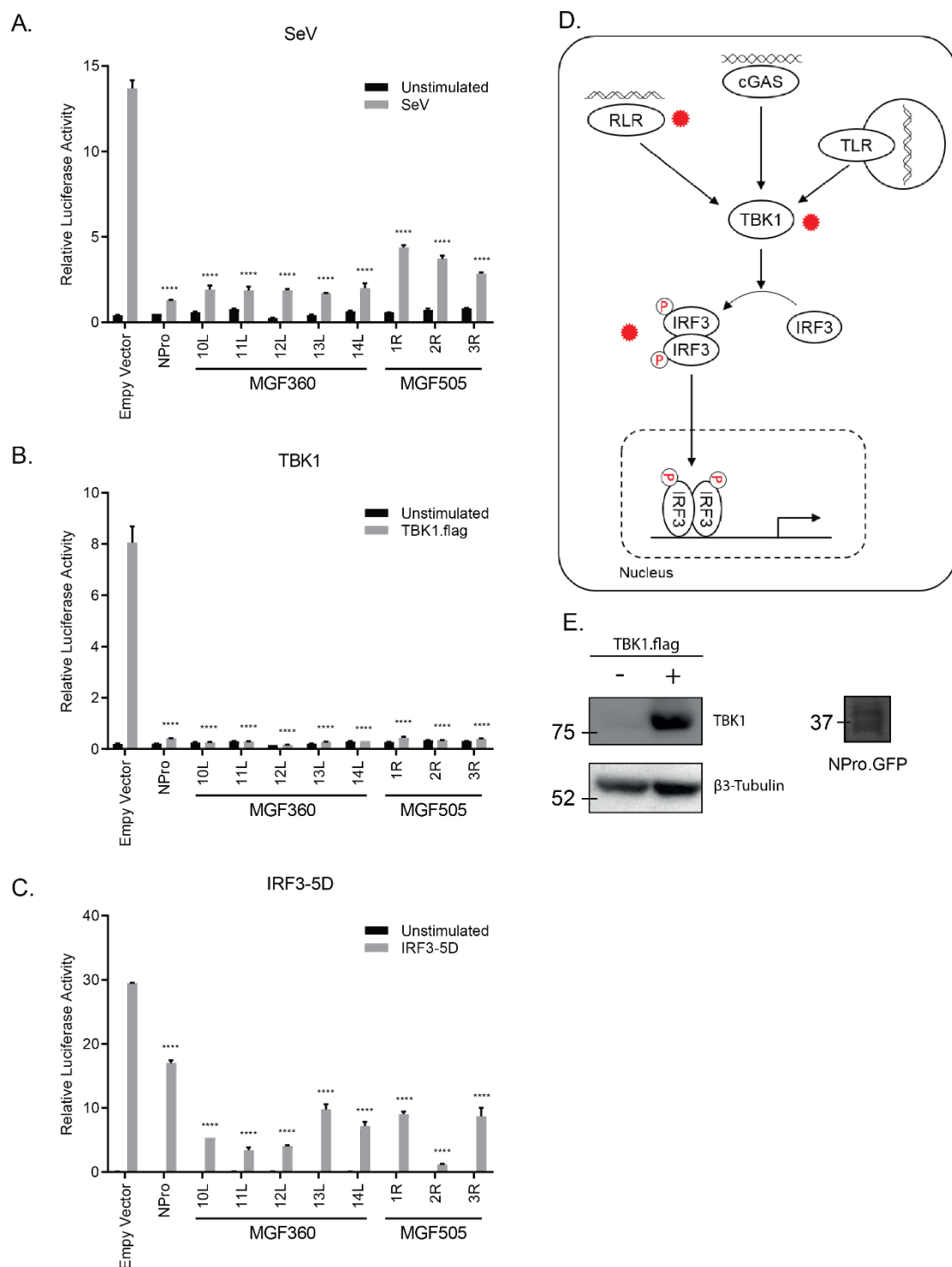


Figure 6-2. Luciferase assay investigating inhibition of the IRF3 pathway by MGF360 and MGF505 proteins HEK293T cells were transfected with luciferase reporter gene under the control of IRF3 regulatory elements of the IFN β promoter. Cell were co-transfected with plasmids expressing the MGF genes indicated fused

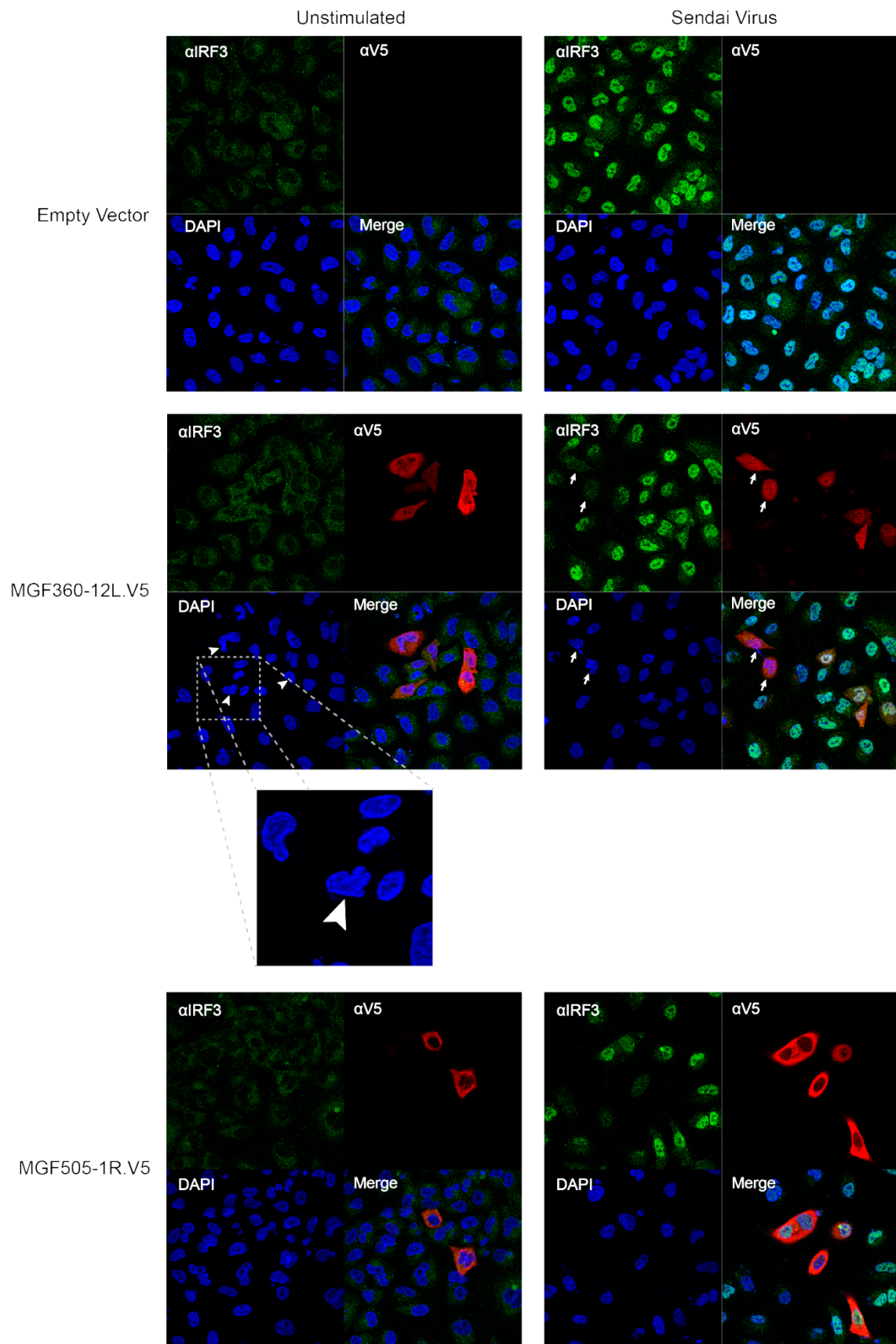
to a V5-tag, or empty vector or NPro as controls. Co-transfection with plasmids expressing renilla luciferase (AB.) or β gal (C.) were used as internal control. Cells were stimulated with: **A.** The ssRNA Sendai virus (200 HAU.mL for 24hours); or transfected to exogenously express **B.** TBK1.flag, or **C.** constitutively active IRF3-5D. **D.** Schematic showing location of stimulation (red star) within the IRF3 pathway. **E.** Western blotting confirming the expression of TBK1.flag, and NPro.GFP. Left hand values indicate the position of molecular weight markers run in parallel in kDa. β 3-Tubulin was used as endogenous loading control. Western blotting is representative of at least duplicate experiments. Luciferase data is representative of three experiments. For statistical analysis two-way ANOVA Tukey's multiple comparison test was performed comparing stimulated Empty Vector to stimulated MGFs. **** = $p \leq 0.0001$, *** = $p \leq 0.001$, ** = $p \leq 0.01$, * = $p \leq 0.05$.

6.2.2 MGF360-12L Partially Inhibits Nuclear Translocation of IRF3

Due to time constraints I decided to focus upon MGF360-12L and MGF505-1R. To observe the effect of MGF360-12L and MGF505-1R on the nuclear translocation of IRF3, A549 cells were transfected with plasmids expressing both proteins fused to V5-tag. Subsequent stimulation of the IRF3 pathway was achieved using Sendai virus (200 HAU.mL) for 4 hours. Immunofluorescent staining was performed to observe V5-tag and IRF3. Nuclear translocation of IRF3 was clearly observed in cells transfected with empty vector pcDNA3.1 control, indicating that the stimulation was sufficient to induce activation of the IRF3 pathway (Figure 6-3A, Empty Vector – Compare Unstimulated vs. Sendai virus panel). 83.3% of cells transfected with empty vector counted (125/150) showed successful activation of the pathway (Figure 6-3B). Interestingly in the presence of MGF360-12L.V5 IRF3 was not observed in the nucleus to the same extent as control cells. A clear trend preventing nuclear translocation was noticed, however this effect was not strong enough to fully abrogate translocation, and furthermore not ubiquitous to all cells expressing the viral protein (Figure 6-3, MGF360-12L.V5 - Compare Unstimulated vs. Sendai virus panels – See arrows). Only 47.5% of cells positively stained for MGF360-12L.V5 counted (28/59) showed IRF3 nuclear translocation, a significant difference compared to control cells (Figure 6-3B). As previously demonstrated in HeLa cells (Figure 5-4), expression of MGF360-12L similarly showed a nuclear and cytoplasmic localisation pattern in A549 cells. It was also noted that a number of cells expressing MGF360-12L displayed a mild nuclear blebbing-like phenotype that was not observed in HeLa cells (Figure 6-3, MGF360-12L.V5 - Compare Unstimulated panel – See arrowheads). Whether MGF360-12L is causing an

impact upon nuclear integrity remains to be seen and warrants further investigation. Staining for MGF505-1R.V5 showed cytoplasmic localisation, in contrast to cytoplasmic and nuclear staining observed in HeLa cells (See Figure 5-4). However unlike with MGF360-12L, a clear inhibition of IRF3 nuclear translocation was not observed (Figure 6-3A, MGF505-1R – Compare Unstimulated to Sendai virus panels). 70.2% of cells positively stained for MGF505-1R.V5 counted (40/57) showed IRF3 nuclear translocation nonsignificant compared to control cells (Figure 6-3B).

A.



B.

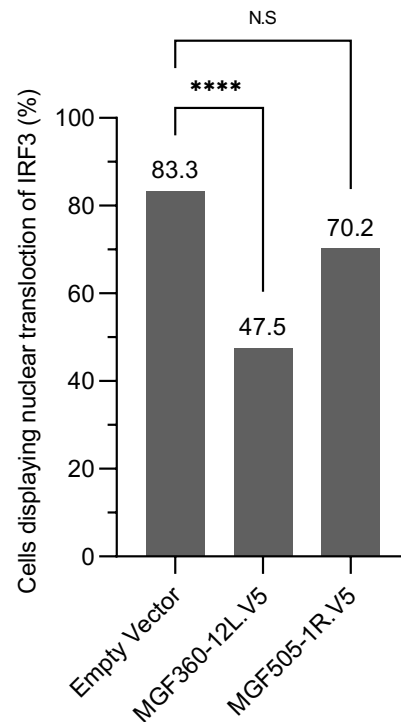


Figure 6-3. MGF360-12L impacted on the nuclear translocation of IRF3 A.

Immunofluorescent staining of transfected A549 cells showing successful translocation of IRF3 to the nucleus upon stimulation with Sendai virus (200 HAU/ml) for 4 hours. Cells were transfected with plasmids expressing MGF360-12L.V5, MGF505-1R.V5 or empty vector control. V5-tag was stained red with mouse anti-V5 antibodies and goat anti-mouse 568 secondary antibodies. IRF3 was stained green with rabbit anti-IRF3, and anti-rabbit 488 secondary antibodies. DAPI was used to stain the nucleus blue. Cells where MGF360-12L was present show a much more limited but not wholly abrogated IRF3 nuclear translocation (**arrows**). A small number of cells expressing MGF360-12L.V5 showed a phenotype of mild nuclear blebbing (**arrowheads**). **B.** Bar chart showing percentage of cells strongly positive for nuclear translocation of IRF3 after Sendai virus stimulation. Percentage is noted at the top of each bar. Number of cells showing IRF3 nuclear

*translocation of total counted - Empty Vector: 125/150 (83.3%), MGF360-12L.V5 : 28/59 (47.5%), MGF505-1R.V5: 40/57 (70.2%). Fisher's exact test was applied for statistical analysis comparing control cells to individual MGFs. **** = $p \leq 0.0001$, *** = $p \leq 0.001$, ** = $p \leq 0.01$, * = $p \leq 0.05$.*

6.2.3 Impact of MGF360-12L & MGF505-1R on Key Components of the IRF3 Pathway

HEK293T cells transfected with MGF360-12L.V5, or MGF505-1R.V5 stimulated with Sendai virus (200 HAU/ml) for 0, 1, 3, or 6 hours. Lysate was analysed by western blotting probing for the presence of TBK1, and IRF3. There was no change in IRF3 amounts with either of the viral proteins (Figure 6-4AB). Curiously, I observed an unexplained dual banding of IRF3 at the correct molecular weight following transfection of cells with MGF505-1R but also with the control empty vector (Figure 6-4B). This has previously been observed in A549 cells, however as to why this occurred in one assay but not another is unexplained (Robitaille, Mariani *et al.* 2016). Interestingly, there appeared to be a slight drop off in the level of endogenous TBK1 in the presence of MGF360-12L, whereas this was not the case for MGF505-1R (Figure 6-4AB), however further quantification of this is needed. From this data I can therefore infer that the inhibition seen is not due to inducing the degradation of IRF3. However, further western blot assessment of an impact upon phosphorylation of IRF3 is required. Additionally, a transfection efficiency lower than 100% could be masking a potential impact of MGF360-12L and MGF505-1R on the endogenous cellular proteins.

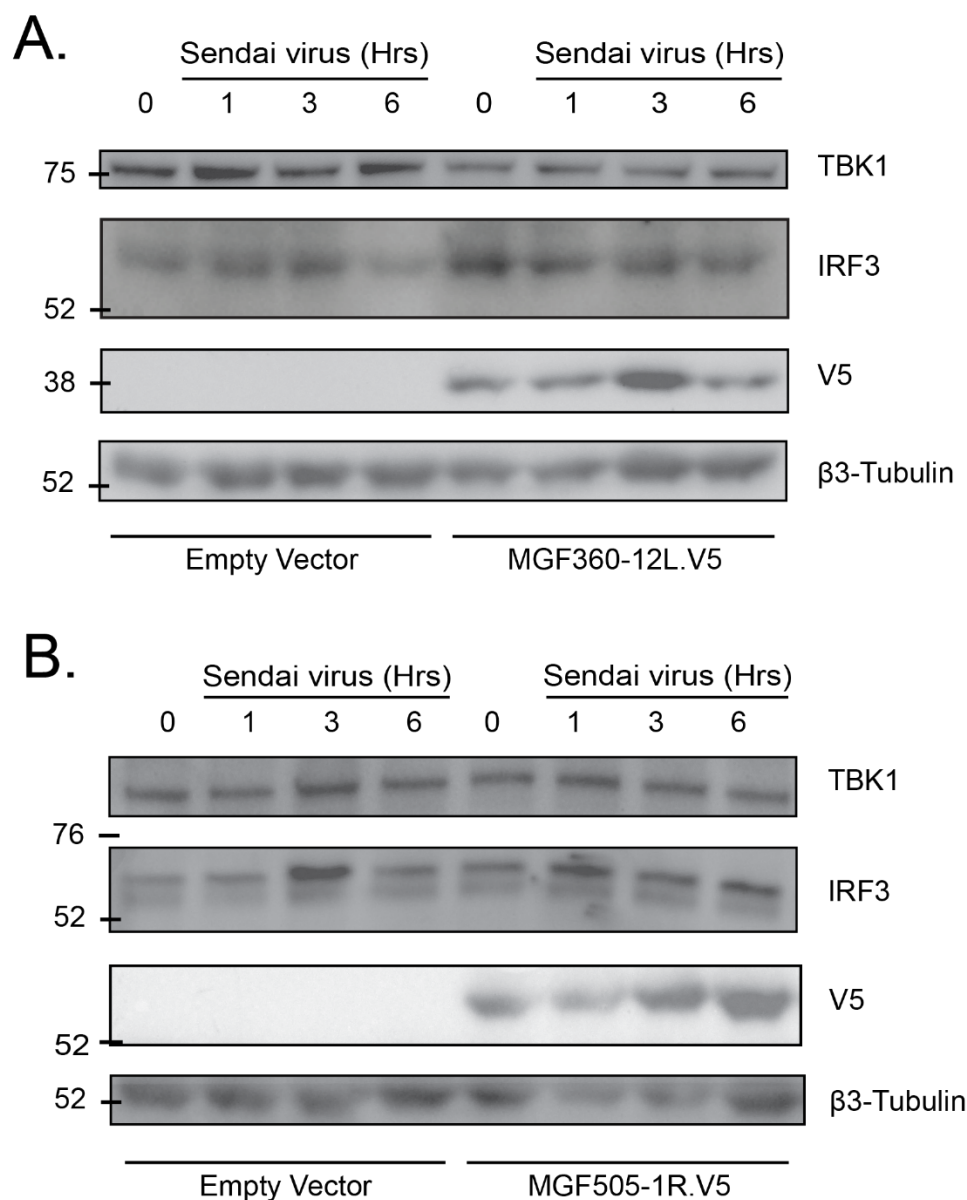


*Figure 6-4. MGF360-12L and MGF505-1R did not appear to impact IRF3 protein levels. Western Blotting of HEK293T cells expressing pcDNA3.1 empty vector and **A.** MGF360-12L.V5. or **B.** MGF505-1R.V5. Cells were stimulated with Sendai virus for different time period in hours indicated in the top bar. Protein levels were investigated by probing with relevant antibodies as indicated on the right-hand side. Left-hand values indicate the position of molecular weight markers run in parallel in kDa. β 3-Tubulin was used as endogenous loading control.*

6.2.4 MGF360-12L did not Co-Immunoprecipitate with Endogenous IRF3

As data thus far has indicated that MGF360-12L is inhibiting the IRF3 pathway at the transcription factor level or below but also has an impact on IRF3 nuclear translocation an obvious point of investigation is whether the viral protein is directly interacting with IRF3. Despite not seeing a reduction of IRF3 by western blotting, a nondegradative interaction with the transcription factor may interrupt IFN β expression by blocking the DNA binding activity of IRF3, or by limiting interactions with cofactors (Wuerth, Habjan *et al.* 2018). Using CoIP as described in 2.7.2, I probed whether MGF360-12L.V5 was interacting with IRF3. HEK293T cells were transfected with plasmid expressing the viral protein fused to a V5-tag, or pcDNA3.1 empty vector was used a control. CoIP was performed in the presence of DSP crosslinker agent. Co-immunoprecipitate was probed for V5-tag, showing successful immunoprecipitation of the viral proteins (Figure 6-5, lane 4). Co-immunoprecipitate and cell lysates were subsequently probed for IRF3. MGF360-12L did not show an interaction with endogenous IRF3 via CoIP (Figure 6-5, lane 4).

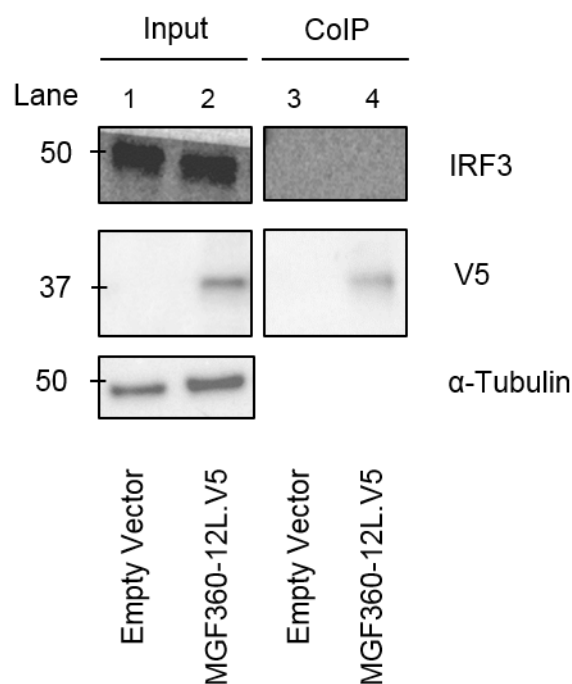


Figure 6-5. CoIP probing for an interaction between MGF360-12L.V5 and endogenous IRF3 HEK293T cells were transfected with plasmids expressing MGF360-12L.V5, or empty vector pcDNA3.1 as control. Cells were incubated in DSP crosslinking agent prior to lysis. Samples of cell lysate were to be used as controls (**Input**). Remaining cell lysates were incubated with magnetic beads covalently linked to mouse anti-V5 antibodies to co-immunoprecipitate V5-tagged viral proteins (Lane 4). **CoIP** lysates were probed for IRF3, and V5 (Lane 4). Left hand values indicate the position of molecular weight markers run in parallel in kDa. α-Tubulin was used as endogenous loading control. The assay was performed in concurrence with CoIP in Figure 5-7, Figure 7-3 and Figure 7-8, therefore, α-Tubulin endogenous control, and V5 probing are the same.

6.2.5 MGF360-12L Inhibits IRF3 Transcriptional Activity in a Luciferase Assay Under the Control of the GAL4 Promoter

As the MGFs appeared to be inhibiting the IRF3 pathway at the transcription factor level or below, I proposed that they may be functioning by inhibiting the transcriptional machinery. Furthermore, as both IRF3 and NF κ B share common components required for the induction of expression of IFN, the previous demonstration (Chapter 5) that these MGFs may be inhibiting the NF κ B pathway via interfering with transcriptional machinery could hint towards a common mechanism of inhibition for both pathways. I tested this hypothesis, by using an alternate β Gal luciferase assay where the expression of firefly luciferase is under the control of the GAL4 promoter. Exogenously expressing Gal4-IRF3-65-427, a fusion protein of the Gal4 DBD and the TAD of IRF3, would stimulate this alternative GAL4 controlled luciferase. Therefore, similar to Gal4DBD-p65 in (See Figure 5-9), stimulation via the exogenous over expression of Gal4-IRF3-65-427 would see induction of firefly luciferase dependent on the IRF3 transcriptional activity but independent of its ability to bind to DNA. HEK293T cells were transfected with plasmids expressing MGF360-10L, 11L, 12L, 13L, 14L, MGF505-1R, 2R, 3R, or Gal4-147 or pcDNA3.1 empty vector as control. Cells were co-transfected with constructs expressing the Gal4-luciferase reporter construct, and β Gal as internal control. Stimulated cells were transfected with Gal4-IRF3-65-427, or unstimulated cells with equal volume of pcDNA3.1 empty vector as controls.

Here I observed no inhibition of luciferase activity in cells transfected with viral genes except for MGF360-12L and Gal127, which both showed significant

decline in the expression of firefly luciferase (Figure 6-6), indicating that MGF360-12L is inhibiting IRF3 transcriptional activity. Curiously however, I unexpectedly saw a significant increased expression in cells co-transfected with MGF360-10L, 13L, 14L and MGF505-2R and 3R (Figure 6-6).

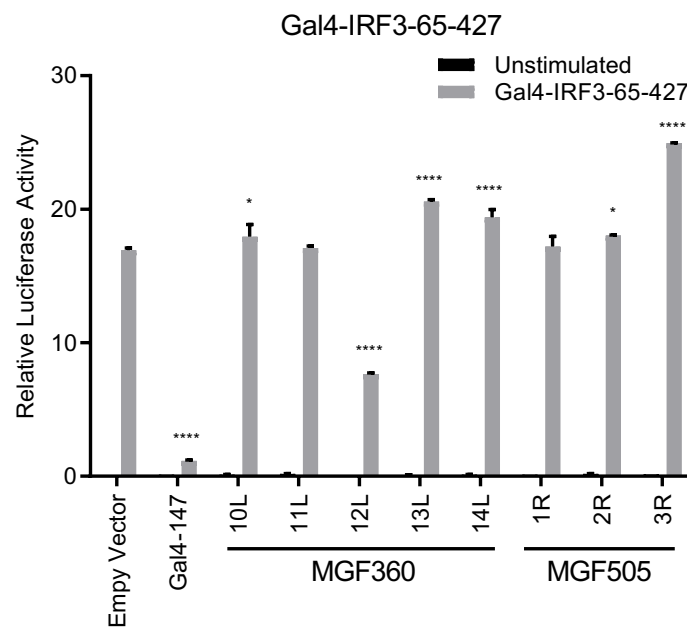


Figure 6-6. Impact of MGFs on transcriptional activity of IRF3 HEK293T cells transfected with constructs expressing Gal4-luciferase reporter gene and β Gal internal control, cells were co-transfected with plasmids expressing V5-tagged MGFs, and stimulated cells transfected with Gal4-IRF3-65-427, or in unstimulated cells co-transfected with equal amounts of pcDNA3.1 empty vector. Data is representative of three experiments. For statistical analysis, two-way ANOVA Tukey's multiple comparison test was performed comparing stimulated Empty Vector to stimulated MGFs. **** = $p \leq 0.0001$, *** = $p \leq 0.001$, ** = $p \leq 0.01$, * = $p \leq 0.05$.

6.3 Discussion

The IRF3 pathway is central in the development of an effective immune response to viral pathogens (See 1.1.4.1). Stimulation of PRRs leads to the activation of the IRF3 transcription factor and the subsequent induction of type-I IFN expression (See 1.1.5). Remarkably the activation of various PRRs all stimulate signalling cascades that utilise an array of unique components yet conjoin at the level of TBK1, the essential kinase in the phosphorylation and thus activation of IRF3 (Fitzgerald, McWhirter *et al.* 2003, Honda and Taniguchi 2006, Panne, McWhirter *et al.* 2007). It is therefore clearly understandable why the IRF3 pathway would be targeted by viral proteins to block an adequate immune response (See 1.1.7). Here I show data indicating that ASFV MGF360-10L, 11L, 12L, 13L, 14L and MGF505-1R, 2R, and 3R are inhibiting the IRF3 pathway at the basal transcription factor level or below (See Table 6-1). This is comparable with the results observed in the NF κ B pathway, perhaps hinting towards a common mechanism.

Using a dual luciferase assay I systematically probed the IRF3 pathway at key steps from PRR, TBK1 kinase and transcription factor level with IRF3-5D. HEK293T cells are not thought to express the endosomal TLR3 therefore Sendai virus driven IRF3 activation was likely stimulated by RLR response (de Bouteiller, Merck *et al.* 2005). Exogenous expression of TBK1.flag was used to stimulate the key kinase directly above the transcription factor in the pathway. Finally in a further attempt to determine the specific location of inhibition, cells were transfected with IRF3-5D, a constitutively active variant of IRF3 with 5 serine

residues mutated to aspartic acid. Results demonstrated that MGF360-10L, 11L, 12L, 13L, 14L, MGF505-1R, 2R and 3R inhibited activation at each of these stages including by expression of IRF3-5D, indicating inhibition of the IRF3 pathway was occurring at the transcription factor below the level of its activation.

MGF	Inhibition of IRF3 dependent luciferase - location	Inhibition of the Gal4-IRF3 65-427 luciferase?	Cellular localization of MGF	Inhibition of IRF3 nuclear translocation?	Other
MGF360-10L	IRF3 activation level or below	No			
MGF360-11L		No			
MGF360-12L		Yes	Cytoplasmic & nuclear – some nuclear blebbing was also observed	Yes	Potential reduction in endogenous TBK1, but not IRF3, was observed.
MGF360-13L		No			
MGF360-14L		No			
MGF505-1R		No	Cytoplasmic	No	No change in endogenous TBK1 or IRF3 level was observed.
MGF505-2R		No			
MGF505-3R		No			

Table 6-1. Overview of the results of the impact of the MGFs on the IRF3 signalling pathway

Due to time, and financial constraints further work was focused upon MGF360-12L and MGF505-1R. Due to inhibition of the IRF3-5D, the impact of MGF360-12L and MGF505-1R on nuclear translocation of IRF3 was investigated using immunofluorescent staining and confocal microscopy. Interestingly this revealed that in A549 cells stimulated with Sendai virus, MGF360-12L partially but inhibited the nuclear translocation of IRF3 by roughly half, a significant reduction relative to control cells. Conversely MGF505-1R only had a marginal impact upon the pathway, and IRF3 appear retained its ability to translocate to the nucleus (Figure 6-3). Regarding MGF505-1R I saw a predominantly cytoplasmic cellular localisation phenotype in A549 which would imply the mechanism of action is non-nuclear, this was not the case in expression of MGF505-1R HeLa cells where localisation was observed in both the cytoplasm and the nucleus (Figure 5-4). As luciferase assays showing inhibition were performed in HEK293T cells (Figure 6-2), whether the inhibitory effect remains intact in A549 cells remains to be seen. Cell line variation therefore may explain this phenotypic difference. Additionally, western blotting indicated that neither viral protein impacted levels of endogenous components of the pathway, however whether transfection efficiency was sufficient to see an effect remains to be seen. The data shown here overall indicates that in the presence of either MGF360-12L or MGF505-1R the functionality of IRF3 activation remained intact (or at least partially in the case of MGF360-12L), thus hinting towards a mechanism of interaction at a level beneath the transcription factor. The Gal4 luciferase system was used for my initial investigation into the potential functional mechanism of proteins from the MGF360 and MGF505 clusters. IRF3-TAD fused to Gal4DBD was used to induce stimulation of the Gal4 luciferase reporter, therefore essentially acting as a model

pathway in place of the PRDI/III region driven luciferase assay. This revealed that relative to the empty vector control, only MGF360-12L could inhibit the reporter assay, indicating a different functional mechanism to the other 7 ASFV genes investigated, similar to the effect seen in Figure 5-9. As expression of the system is induced by the Gal4 binding domain binding to the GAL4 promoter and the IRF3 TAD driving expression, I can thus infer that MGF360-12L is interfering with IRF3 TAD or transcriptional activity. Phosphorylation of serine 386 residue of the C-terminus of the IRF3 TAD allows the induction of dimerisation, and combined with phosphorylation at serine 396 and formation of an acidic surface binding to crucial transcriptional cofactors CBP/p300 is enabled (Lin, Génin *et al.* 2000, Taniguchi, Ogasawara *et al.* 2001, Honda and Taniguchi 2006, Chen, Srinath *et al.* 2008, Takahasi, Horiuchi *et al.* 2010). I therefore attempted to assess the impact of MGF360-12L and MGF505-1R on the phosphorylation status of IRF3 at different sites via western blotting, however this was not successful (data not shown). The inhibition of MGF360-12L on IRF3 transcriptional activity may be indirect via transcriptional cofactors. Gates and Shisler (2016) used IRF3 controlled luciferase assays to demonstrate that the cellular cFLIP long isoform variant cFLIP_L imposed a strong inhibitory function on the pathway stimulated by exogenous IRF3-5D, thus the impact was occurring beyond IRF3 activation. The authors revealed that cFLIP_L interacted with IRF3 which correlated with a loss of IRF3-CBP interaction, and this was responsible for IRF3 inhibition (Gates and Shisler 2016). However, it is of note that these interaction experiments were performed with exogenous expression of cFLIP_L, IRF3 and CBP. Further work examining whether MGF360-12L disrupts IRF3's interaction with CBP or other cofactors is obviously required. EMSA assay may offer insight as to whether IRF3

is binding to target promoter site in the presence of the viral protein, whereas ColP would offer a potential route of investigation to determine if IRF3-CBP interactions are affected in the presence of this MGF protein.

Due to the obvious criticality of IRF3 in mounting an appropriate response to pathogen, numerous viruses have been demonstrated to deploy tactics targeting IRF3 directly to subvert the innate immune response (Hilton, Moganeradj *et al.* 2006, Bauhofer, Summerfield *et al.* 2007, Seago, Hilton *et al.* 2007, Sen, Feng *et al.* 2009). The classical swine fever virus protein NPro has been shown to target IRF3 for proteasomal degradation (Bauhofer, Summerfield *et al.* 2007, Seago, Hilton *et al.* 2007). Using PK15 cells stably expressing NPro, used here as a positive control, Seago, Hilton *et al.* (2007) revealed that in the presence of the viral protein there was a remarked decrease in IRF3 level. This work was confirmed by Bauhofer, Summerfield *et al.* (2007) who further revealed that NPro coprecipitates with IRF3, and suggested that this interaction is responsible for proteasomal degradation of the transcription factor. Interestingly Hilton, Moganeradj *et al.* (2006) proposed that nuclear IRF3 is resistant to NPro mediated degradation but is in fact prevented from binding to promoter DNA. NSP1 protein of virulent rotavirus strains RRV directly degrades IRF3 in a cell dependent manner to subdue the early IFN response in order to enhance viral replication (Barro and Patton 2005, Sen, Feng *et al.* 2009). Epstein-Barr virus BGLF4 protein directly interacts with IRF3 preventing poly I:C stimulated IFN β mRNA expression, but retained its ability to dimerise, translocate to the nucleus, and further recruit CBP. Chromatin immunoprecipitation showed that BGLF4 in

fact prevented recruitment of IRF3 to the $\text{INF}\beta$ promoter (Wang, Doong *et al.* 2009). Therefore, there is precedent of viral proteins directly interacting with IRF3 to subvert the pathway. Despite clear inhibition of the pathway at the level of IRF3 or below, I was unable to observe any obvious impact on the actual functionality of the pathway, with the notable exception of partial inhibition of IRF3 nuclear translocation by MGF360-12L. However, I did not see a direct interaction between IRF3 and MGF360-12L (Figure 6-5). This may provide indication that the mechanism of inhibition is occurring independently of the transcription factor. Numerous viral proteins have been shown to inhibit the IRF3 pathway via other mechanisms. For example, SARS-CoV1 PLpro was initially implicated in inhibiting IRF3 via preventing its phosphoactivation (Devaraj, Wang *et al.* 2007, Frieman, Ratia *et al.* 2009). However, luciferase assays revealed that PLpro inhibits the constitutively active IRF3-5D and thus inhibits IRF3 post activation, but does not impact upon nuclear translocation or dimerisation, nor directly interact with the transcription factor, hinting towards inhibition of a multifunctional nature (Frieman, Ratia *et al.* 2009, Matthews, Schäfer *et al.* 2014). Mutagenesis showed that this papain-like protease protein utilises a mechanism of blocking the pathway dependent upon its de-ubiquitination activity, ultimately preventing IRF3 driven gene expression (Matthews, Schäfer *et al.* 2014). Sicilian sandfly virus NSs does not block IRF3 activation but interacts with the transcription factor's DBD limiting its interaction with the promoter (Wuerth, Habjan *et al.* 2018). Additionally, cofactors CBP and p300 have shown to be common targets. Tick-borne Thogoto virus ML protein prevents CBP-IRF3 interaction, preventing recruitment of RNAPII to the IFN promoter (Vogt, Preuss *et al.* 2008, Haas, Meiler

et al. 2018). Significant further examination is required to fully understand in the inhibition of the MGFs on the IRF3 pathway.

The data here demonstrates that members of the MGF360 and MGF505 clusters show a strong inhibitory function of the IRF3 pathway at the transcription factor level or below, and begins to provide significant further clues for routes of investigation to reveal the specific mechanistic function of the MGFs with a specific focus on MGF360-12L and MGF505-1R proteins.

7 UNDERSTANDING THE MECHANISM(S) OF ACTION OF MGF360-12L & MGF505-1R DRIVEN INHIBITION OF THE NF κ B AND IRF3 PATHWAYS

7.1 Introduction

Here I will describe investigations aiming to understand the specific mechanism(s) of inhibition of the NF κ B and IRF3 pathways by MGF360-12L and MGF505-1R.

As previously described in 1.1.7.4, the closely related SOCS-Box and VHL-Box motifs are responsible for the recruitment of CRL E3 Ub ligase machinery via EloB EloC heterodimer, and a Cul2 or 5 scaffold proteins depending on motif (Figure 1-3) (Kamura, Maenaka *et al.* 2004). I previously demonstrated that the MGF360 and MGF505 family of proteins contain what appear to be novel VHL/SOCS-Box-like motifs, although specifically which of these two motifs was not initially obvious from sequence alone (See 3.2.4). Here I investigate the functionality of the motifs and their relevance to the inhibitory activity of these MGFs on the NF κ B and IRF3 pathways. As I previously showed both IRF3 (Chapter 5) and NF κ B (Chapter 6) pathways are inhibited by the MGFs at the level of the transcription factor level or below, it was a logical step to investigate common components of both pathways. As the expression of IFN- α/β can be driven by the interaction of both transcription factors with the IFN promoter this was an obvious initial route of examination. The IFN- α/β promoter can be divided into PRD regulatory

elements. PRDI and III are binding sites for IRF3, whereas PRDII for NFκB (Figure 7-1A). As previously described (See 1.1) infection leads to activation and nuclear translocation of these key transcription factors where they bind to κB (NFκB), or ISRE (IRF3) sites within the PRDs inducing transcription (Kim and Maniatis 1997). A further architectural chromosomal protein high-mobility group protein I(Y) (HMGI(Y)) facilitates binding of the NFκB transcription factor (Thanos and Maniatis 1992, Kim and Maniatis 1997). Together this higher-order nucleoprotein complex is known as the “enhanceosome” (Figure 7-1B). However, binding of the transcription factor to these regions of the promoter alone is insufficient for transcription initiation, but in fact requires the further recruitment of proteins to the complex (Merika, Williams *et al.* 1998). This includes histone acetyl transferase proteins such as CBP, or its paralog p300. These co-activators aggregate directly at the promoter with the enhanceosome targeting H3H4 histones for acetylation, and are considered essential for transcriptional synergy (Figure 7-1C) (Gerritsen, Williams *et al.* 1997, Merika, Williams *et al.* 1998, Yoneyama, Suhara *et al.* 1998, Qin, Liu *et al.* 2005, Mukherjee, Behar *et al.* 2013).

CBP/p300 are versatile co-activators that offer a promiscuous approach to the induction of transcription as they are capable of forming complexes with a wide variety of transcription factors, including NFκB, IRF3, IRF7, AP-1, c-Jun, C-Fos amongst others (Janknecht and Hunter 1996, Gerritsen, Williams *et al.* 1997, Yoneyama, Suhara *et al.* 1998, Yang, Lin *et al.* 2003, Qin, Liu *et al.* 2005, Mukherjee, Behar *et al.* 2013). The protein domains facilitating the interaction between a specific transcription factor and CBP/p300 are highly varied, with no obvious

similarities. Qin, Liu *et al.* (2005) revealed that IRF3-CBP/p300 interaction is mediated by hydrophobic elements of the IRF3 IAD domain of IRF3 and IRF3-Binding domain of CBP. Upon activation of the IRF3 pathway, phosphorylation and dimerisation of IRF3 facilitates the interaction with CBP/p300 and in fact strongly increases the affinity of IRF3 binding to promoter DNA (Yang, Lin *et al.* 2002). The p65 subunit of NFκB however, forms interactions with either the KIX or TAZ1 domain of CBP/p300, via the transcription factor's DBD and TAD respectively (Gerritsen, Williams *et al.* 1997, Mukherjee, Behar *et al.* 2013). Moreover, interaction between p65 or IRF3, and CBP/p300, is crucial in increasing transcription factor dependent promoter activation (Gerritsen, Williams *et al.* 1997, Yoneyama, Suhara *et al.* 1998).

Transcription is also dependent upon recruitment of a number of other proteins including RNA polymerase and general transcription factors. RNAPII, is a large holoenzyme multiprotein complex that catalyses transcription. It is formed of a multiple subunits, with the largest being RNAPII subunit B1 (Rpb1). Precise RNAPII activity is also dependant on at least 6 general transcription factors, TFIIA, TFIIB, TFIID, TFIIIE, TFIIF, and TFIIH. These general transcription factors assemble with RNAPII and a large number of other proteins to form the PIC at the IFN promoter (Agalioti, Lomvardas *et al.* 2000, Honda, Takaoka *et al.* 2006). TFIIB is crucial to transcription, and functions by interacting with TFIID at a TATA box upstream of the transcription start site and is essential in the recruitment of RNAPII (Figure 7-1D) (Agalioti, Lomvardas *et al.* 2000, Honda, Takaoka *et al.* 2006).

Interestingly the enhanceosome facilitates the recruitment of TFIIB to the promoter, and subsequently RNAPII via CBP/p300 (Kim, Kim *et al.* 1998). Enhanceosome mediated recruitment of TFIIB, and CBP/p300 driven histone acetylation induces the recruitment of the remaining PIC components including RNAPII holocomplex lacking TFIID subunit, along with BAF thus inducing further chromatin remodelling and nucleosome displacement (Kim, Kim *et al.* 1998, Agalioti, Lomvardas *et al.* 2000, Honda, Takaoka *et al.* 2006). This makes the transcription start site available for TFIID, finally allowing transcription to commence (Figure 7-1D)(Agalioti, Lomvardas *et al.* 2000, Honda, Takaoka *et al.* 2006). This crucial transcription machinery common to the expression of IFN in both IFN3 and NFκB pathways may offer potential targets for MGFs, and thus explain the inhibition seen to both signalling cascades.

Here I demonstrate that the putative VHL/SOCS-Box-like motifs of MGF360-12L and MGF505-1R are functional as a VHL-Box, and SOCS-Box respectively, and that both viral proteins interact with the apposite CRL machinery. Furthermore, site-directed mutation of the motif in MGF505-1R disrupts the inhibition of both the NFκB and IRF3 pathways, whereas this was not the case for MGF360-12L which retained its inhibitory function.

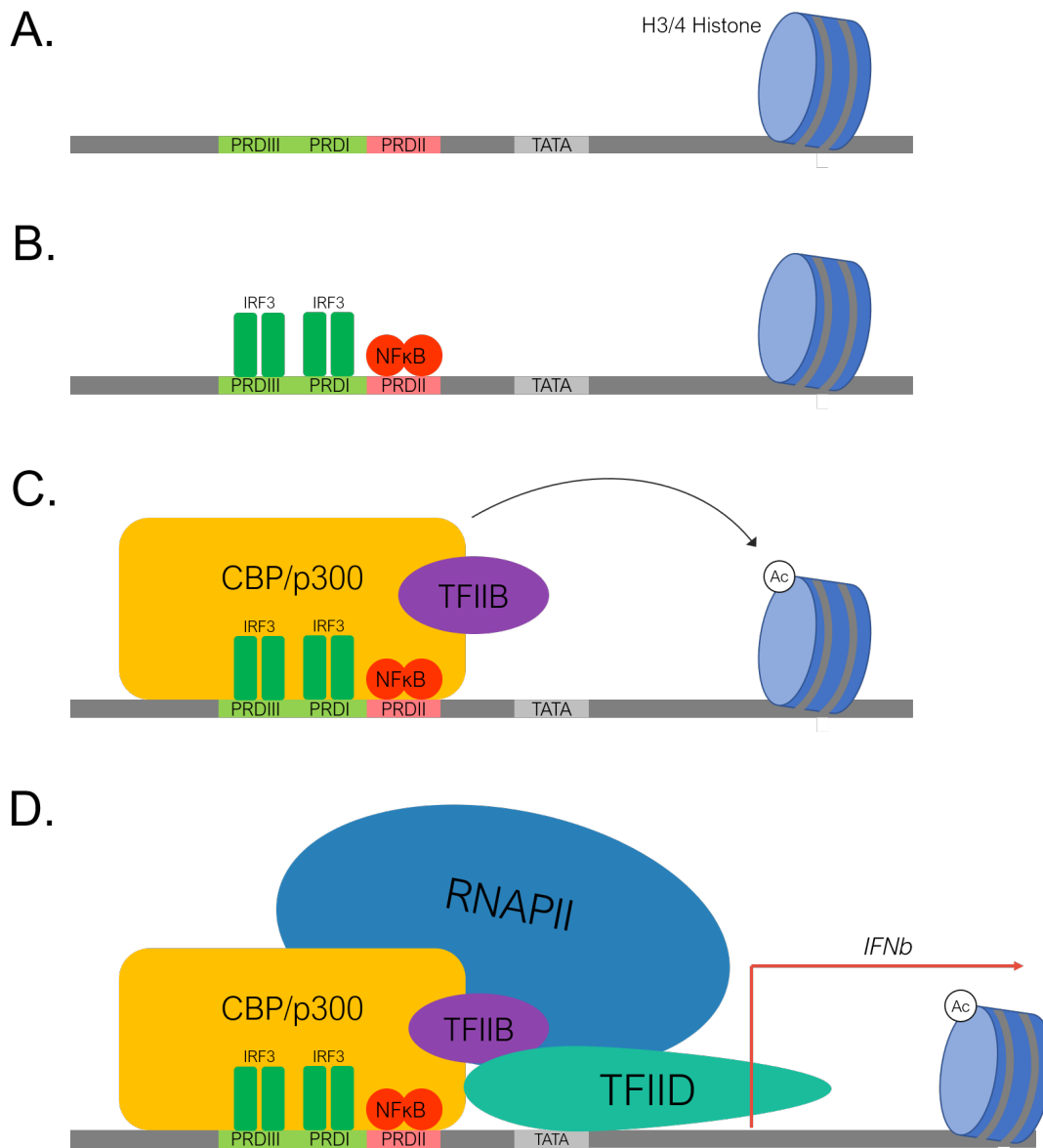


Figure 7-1. Overview of the stages of de novo transcription initiation of the IFN β gene **A.** Promoter region of the gene in unstimulated conditions. **B.** Activation of the NF κ B or IRF3 pathways leads to transcription factor activation, nuclear translocation, and binding to respective regions of the IFN promoter. **C.** Formation of the enhanceosome. Recruitment of cofactors such as CBP/p300, and subsequent recruitment of general transcription factor TFIIB. Acetylation of histone induces chromatin remodelling. **D.** Recruitment of RNAPII and finally prior chromatin remodelling allowing TFIID binding to induce transcription initiation. Schematic adapted from Honda, Takaoka et al. (2006).

7.2 Results

7.2.1 Generation of MGF360-12L & MGF505-1R Mutants

I discovered that members of MGF505 and MGF360 clusters have a putative novel N-terminal VHL/SOCS-Box-like motif, consisting of a BC-Box and an undetermined Cul2 or Cul5-Box (See 3.2.3). In order to assess the functionality of the motif in MGF360-12L and MGF505-1R, I mutated key residues to investigate its importance in the NFκB and IRF3 inhibitory function of the viral proteins (Figure 7-2). Residues described as key for the functionality of the motif were targeted for mutation to alanine with the aim of disrupting functionality (Figure 7-2) (Kamura, Maenaka *et al.* 2004, Mahrour, Redwine *et al.* 2008). I generated 3 mutant variants for MGF360-12L and MGF505-1R genes as described in 2.4.7, with sequences coding for key residues mutated in the full motif (MGF360-12L.mVHL.V5, MGF505-1R.mSOCS.V5), BC-Box alone (MGF360-12L.mBC.V5, MGF505-1R.mBC.V5) or the Cul-Box alone (MGF360-12L.mCUL.V5, MGF505-1R.mCUL.V5) (Figure 7-2). Mutant variants of the genes coding for the proteins were cloned into pcDNA3.1 vector and fused to a C-terminal V5-tag.

As the BC-Box of MGF360-12L was partially conserved, only residues that matched the key residues of the motif were mutated. Furthermore, as it was unclear as to whether the Cul-Boxes were either a Cul2 or a Cul5-Box, mutations were introduced to cover all residues that matched either of the motifs (Figure 7-2A.). It is also of note that in MGF505-1R mutants, residues adjacent to key residues of the motif were also mutated to alanine (Figure 7-2B.). This was due

to these residues being highly conserved across all MGF505 paralogues of Georgia07/1 isolate proteins.

A.

	BC-Box										Cul2/5-Box																																					
	t					c																																										
	s					a																																										
VHL/SOCS-Box	---pL-----s---φ-----φ-----LPφP-----φ---φ---																																															
MGF360-12L.V5	MLP	SL	Q	S	L	T	K	K	V	L	A	G	Q	C	V	P	T	N	Q	H	Y	L	L	K	C	Y	D	L	W	W	H	D	A	P	I	T	E	D	H	N	I	R	L	T	K	S	A	50
MGF360-12L.mVHL.V5	MLP	AA	Q	S	L	T	K	K	V	A	A	G	Q	C	V	P	T	N	Q	H	Y	L	L	K	C	Y	D	A	W	W	H	D	AA	I	T	A	D	H	N	I	R	L	T	K	S	A	50	
MGF360-12L.mBC.V5	MLP	AA	Q	S	L	T	K	K	V	A	A	G	Q	C	V	P	T	N	Q	H	Y	L	L	K	C	Y	D	L	W	W	H	D	AA	I	T	A	D	H	N	I	R	L	T	K	S	A	50	
MGF360-12L.mCUL.V5	MLP	SL	Q	S	L	T	K	K	V	L	A	G	Q	C	V	P	T	N	Q	H	Y	L	L	K	C	Y	D	A	W	W	H	D	AA	I	T	A	D	H	N	I	R	L	T	K	S	A	50	

B.

	BC-Box												Cul2/5-Box																													
	t	c																																								
	s	a																																								
VHL/SOCS-Box	--pL---	s---	φ---	LPφP---	φ---	φ---	φ---																																			
MGF505-1R.V5	MFS	LQNL	CRKT	L	PNRK	L	E	E	F	F	D	E	Y	I	L	Q	L	L	G	L	Y	W	E	N	H	G	T	I	Q	R	A	G	N	N	C	V	L	I	Q	50		
MGF505-1R.mSOCS.V5	MFA	AAAA	AA	AKT	A	PNR	K	AA	E	F	F	A	D	E	Y	A	L	Q	L	L	G	L	Y	W	E	N	H	G	T	I	Q	R	A	G	N	N	C	V	L	I	Q	50
MGF505-1R.mBC.V5	MFA	AAAA	AA	AKT	A	PNR	K	L	E	E	F	F	D	E	Y	I	L	Q	L	L	G	L	Y	W	E	N	H	G	T	I	Q	R	A	G	N	N	C	V	L	I	Q	50
MGF505-1R.mCUL.V5	MFS	LQNL	CRKT	L	PNR	K	AA	E	F	F	A	D	E	Y	A	L	Q	L	L	G	L	Y	W	E	N	H	G	T	I	Q	R	A	G	N	N	C	V	L	I	Q	50	

Figure 7-2. Mutations of the putative VHL/SOCS-Boxes of MGF360-12L and MGF505-1R Schematic showing sequences of putative SOCS/VHL-Box-like in **A**. MGF360-12L and **B**. MGF505-1R, and corresponding mutations of said motif with the aim of impairing functionality. Residues highlighted in yellow correspond to MGF residues that are homologous with key residues of the BC-Box, highlighted green corresponds to Cul2-Box, and pink residues or residues highlighted in pink correspond to key Cul5-Box residues. Residues in bold are mutations.

7.2.2 MGF360-12L Co-Immunoprecipitates with Endogenous VHL CRL Machinery

CoIP was used to investigate interacting partners of MGF360-12L as described in 2.7.2. HEK293T cells were transfected with pcDNA3.1 Empty Vector control plasmid, wild-type MGF360-12L.V5, or mutant variant with mutations in key residues of the putative BC-Box and Cul-Box of the VHL/SOCS-Box-like motif, MGF360-12L.mVHL.V5 (Figure 7-2A). Cells were incubated in DSP crosslinking agent prior to cell lysis. On the exogenous expression of MGF360-12L.V5, and mutant variant MGF360-12L.mVHL.V5, by using epoxy magnetic beads covalently linked to anti-V5 antibodies I was able to co-immunoprecipitate the expressed V5-tagged viral proteins (Figure 7-3 - Lane 5 & 6). This was not possible in the absence of DSP (data not shown). DSP is a membrane permeable crosslinker that contains terminal amine-reactive N-hydroxysuccinimide esters. These terminal esters typically target amines of lysine residues, allowing the formation of 12.0Å crosslinks between proteins. Cross link formation is reversible with the disulphide bridges being cleaved by incubation in reducing agent DTT. Samples were incubated in DTT to cleave the linker prior to SDS-PAGE separation. Input was probed for V5-tag expressing viral proteins, and potential interacting partners to confirm their expression. MGF360-12L.V5, MGF360-12L.mVHL.V5, Cul2, Cul5, EloB and EloC were all expressed at the expected molecular weights (Figure 7-3 – Input Lanes 1-3). In the presence of DSP MGF360-12L.V5 pulled down endogenous EloB, EloC, and Cul2 at the expected molecular weights (15kDa, 13kDa, and 75kDa) (Figure 7-3 – CoIP Lane 5). Precipitation of interacting partners was not observed in lysates from cells

transfected with empty vector, nor mutant variant MGF360-12L.mVHL.V5 suggesting the VHL-Box motif is required for the interaction (Figure 7-3 - Lane 4 and 6). It is of importance to note that Cul5, a key component in the generation of SOCS-Box machinery, was observed in the input at the correct molecular weight (approximately 80KDa), but not observed in the CoIP, suggesting that the motif is indeed a VHL-Box, and not a SOCS-Box (Figure 7-3 – Lanes 1-3). From here onwards, the VHL/SOCS-Box motif of MGF360-12L will thus be referred to as a VHL-Box.

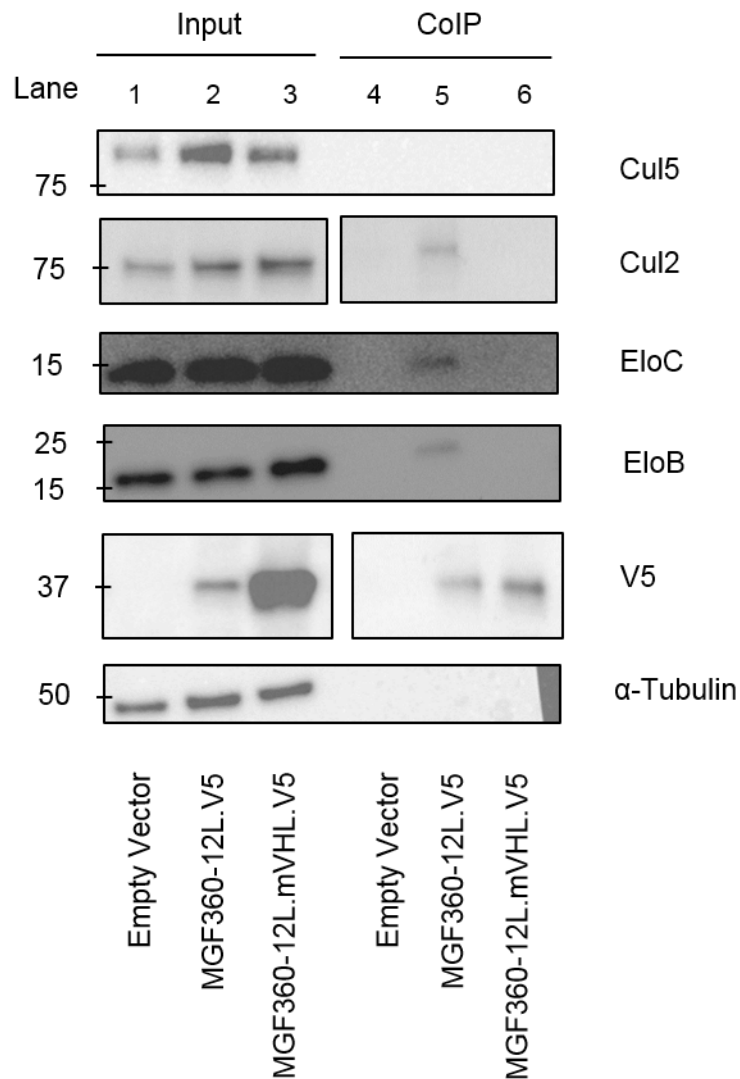


Figure 7-3. CoIP probing for interacting partners of MGF360-12L Lysate of HEK293T cells transfected with empty vector (Lane 1 and 4) or, V5-tagged viral genes (MGF360-12L.V5 (Lane 2 and 5), MGF360-12L.mVHL.V5 (Lane 3 and 6)). All cells were incubated in DSP cross linker prior to lysis. Sample of the lysate was taken as **Input** (Lanes 1-3). The remaining lysate was incubated with magnetic beads covalently linked to anti-V5 antibodies to co-immunoprecipitate viral protein (**CoIP**) (Lanes 4-6). Samples were analysed by western blotting following SDS-PAGE separation. Bands corresponding to V5-tag, Cul2, Cul5, EloB and EloC were present in the input (Lanes 1-3). CoIP was probed for V5, Cul2, Cul5, EloB and EloC (Lanes 4-6). Bands corresponding to V5-tagged MGF360-12L.V5 and MGF360-

12L.mVHL.V5 were observed in the ColP at the correct molecular weight (Lanes 5 and 6). Bands corresponding to Cul2, EloB and EloC were also detected at the correct predicted molecular weight in pulldowns with MGF360-12L.V5 (Lane 5), but not in the empty vector control (Lane 4) or mutant variant samples (Lane 6). Molecular weight protein ladder was run in parallel with samples, and KDa are noted on the left-hand side. α -Tubulin was used as endogenous loading control. Data is representative of experiments performed in duplicate. The assay was performed in concurrence with ColP in Figure 5-7, Figure 6-5, and Figure 7-8. Therefore α -Tubulin endogenous control, and V5 probing is the same.

7.2.3 MGF05-1R Co-Immunoprecipitates with SOCS CRL Machinery

A similar approach to 7.2.2 was used to probe for interactions between MGF505-1R protein and Cul2, Cul5, EloB and EloC. HEK293T cells were transfected with pcDNA3.1 empty vector as control, wild-type MGF505-1R.V5, or mutant variant MGF505-1R.mSOCS.V5 with key residues of both BC-Box and Cul2/5-Box of the putative SOCS-Box disrupted (Figure 7-2B). Unlike with MGF360-12L, I observed co-precipitation between MGF505-1R and SOCS-Box machinery. By using DSP crosslinker to stabilise protein interactions I was able to successfully immunoprecipitate the V5-tagged wild-type viral protein MGF505-1R.V5 and mutant variant MGF505-1R.mSOCS.V5 using epoxy beads covalently linked with anti-V5 antibodies (Figure 7-4A - Lane 5 & 6 – V5). Using antibodies targeting endogenous Cul2, Cul5, EloB and EloC proteins, I interestingly observed CoIP of MGF505-1R.V5 and EloB, but was unable to pulldown EloC, Cul2 or Cul5 (Figure 7-4A - Lane 5). I was unable to immunoprecipitate any interacting proteins from cells transfected with the empty vector control (Figure 7-4A - Lane 3), or the mutant variant MGF505-1R.mSOCS.V5 (Figure 7-4A – Lane 6). However, following co-transfection into HEK293T cells of plasmids expressing Cul2.Myc and Cul5.Myc, MGF505-1R.V5 co-precipitated with Cul5.Myc but not Cul2.Myc (Figure 7-4B and C - Lane 5). This was unexpected as the putative Cul-Box motif was more reminiscent of a Cul2 binding region than that of Cul5 (See 3.2.4). Wild-type protein and MGF505-1R.mSOCS.V5 did not co-precipitate with Cul2.Myc (Figure 7-4B - Lane 5 and 6). However, a very faint band could be observed with Cul5.Myc (Figure 7-4C - Lane 6), although this may be explained by over expression of the protein forcing the interaction with the mutant protein, or that the mutation did not fully abrogate the Cul5 binding motif and some residual

interaction may still be occurring. Additionally, the pulldown of the MGF505-1R.mSOCS.V5 protein itself was weak, compared to wildtype, therefore relatively less Cul2 may have been coimmunoprecipitated, making the result appear as a weaker interaction. Further work is required to explain and quantify these results. In an attempt to observe an interaction between MGF505-1R.V5 and EloC, I transfected a plasmid expressing EloC fused to a T7 tag, together with MGF505-1R.V5 and MGF505-1R.mSOCS.V5. I was unable to co-immunoprecipitate either wild-type or mutant MGF505-1R with overexpressed EloC (Figure 7-4D - Lane 5 & 6). However, taken together this revealed that the SOCS/VHL-Box-like motif in MGF505-1R is likely functioning as SOCS-Box.

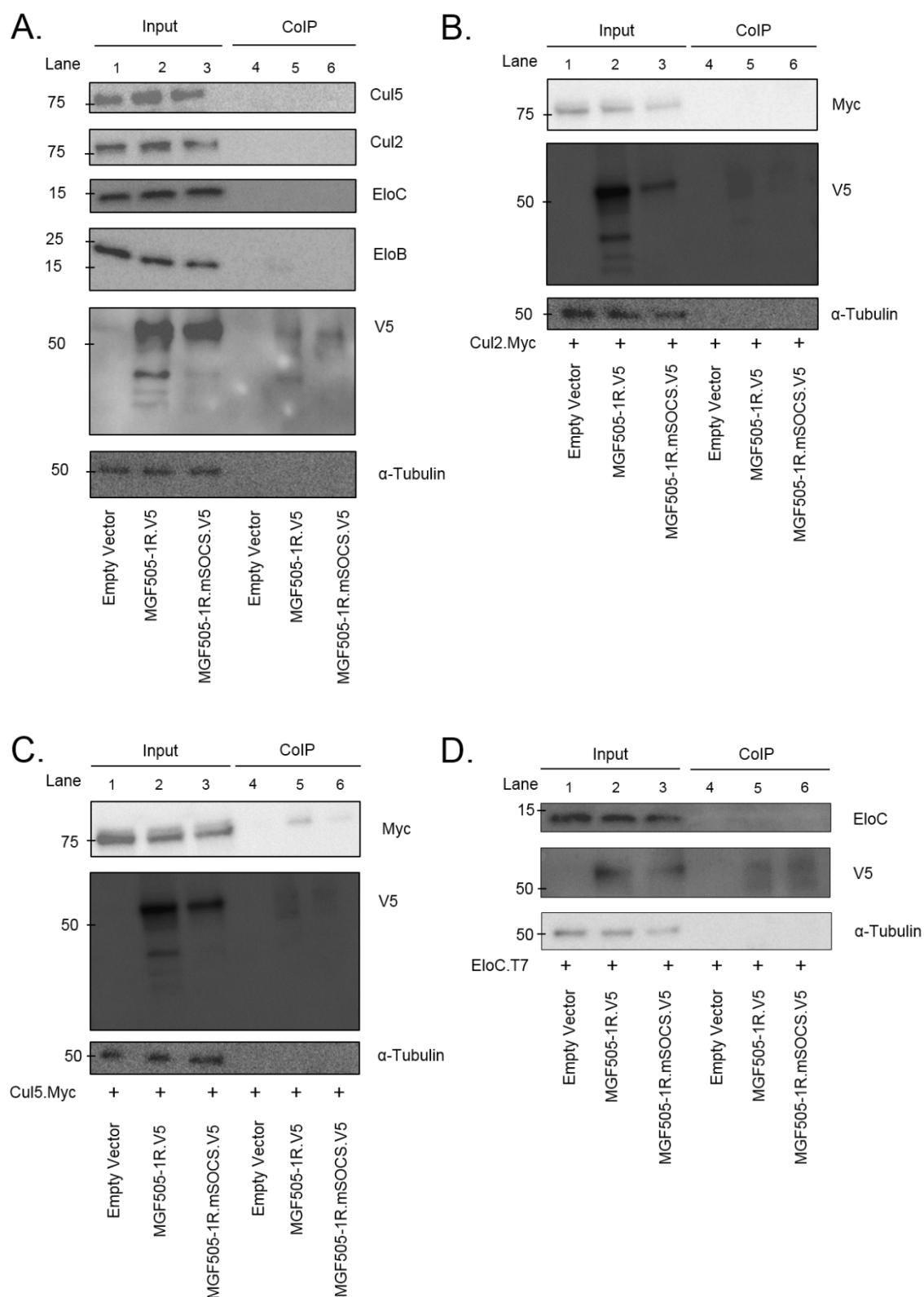


Figure 7-4. CoIP probing for interacting partners of MGF505-1R **ABCD.** Lysates of HEK293T cells transfected with empty vector (Lane 1 and 4) or, V5-tagged viral genes MGF505-1R.V5 (Lane 2 and 5), MGF505-1R.mSOCS.V5 (Lane 3 and 6)). All

cells were incubated in DSP cross linker prior to lysis. A sample of the lysate was taken as "Input" (Lanes 1-3). The remaining lysate was incubated with magnetic beads covalently linked to anti-V5 antibodies to pulldown V5-tagged proteins (CoIP - Lanes 4-6). Samples were analysed by western blotting following SDS-PAGE separation. Bands corresponding to V5-tagged MGF505-1R.V5 and MGF505-1R.mSOCS.V5 were observed in the Input (Lanes 2 and 3) and CoIP (Lanes 5 and 6) at the correct molecular weight. **A.** Input and CoIPs were probed with antibodies against endogenous Cul2, Cul5, EloB and EloC. **B.** Cells were additionally co-transfected with plasmids expressing Cul2.Myc. Input and CoIP were probed with anti Myc-antibodies. **C.** Cells were additionally co-transfected with plasmids expressing Cul5.Myc. Input and CoIP were probed with anti-Myc antibodies. **D.** Cells were additionally co-transfected with plasmids expressing EloC.T7. Input and CoIPs were probed with anti-EloC antibodies. Molecular weight protein ladders were run in parallel and their position in KDa are noted on the left-hand side. α -Tubulin was used as endogenous loading control. CoIP in **A** was performed in concurrence with CoIP in Figure 5-8 and Figure 7-7B therefore, α -Tubulin endogenous control, and V5 probing is the same.

Interactor		MGF360-12L	MGF505-1R
EloB	Endogenous	+	+
	Exogenous	Not Tested	Not Tested
EloC	Endogenous	+	-
	Exogenous	Not Tested	-
Cul2	Endogenous	+	-
	Exogenous	Not Tested	-
Cul5	Endogenous	-	-
	Exogenous	Not Tested	+
SOCS-Box or VHL-Box?		VHL-Box	SOCS-Box

Table 7-1. Overview of the observed interacting partners of the VHL/SOC-Box CRL machinery with MGF360-12L, and MGF505-1R + indicates an interaction was observed, whereas – indicates no interaction.

7.2.4 Mutation of MGF505-1R SOCS-Box Alleviated Inhibition of the NF κ B & IRF3 Pathways, but Mutation of MGF360-12L VHL-Box did not

Using dual/ β Gal luciferase assays as described in 2.6, I investigated the impact of introducing mutations into the VHL-Box of MGF360-12L, and SOCS-Box of MGF505-1R on inhibition of the NF κ B and IRF3 pathways. HEK293T cells were transfected with plasmids containing firefly luciferase reporter gene under the control of either IRF3 or NF κ B regulatory elements of the IFN- β promoter, and plasmids expressing β Gal (for IRF3-5D stimulation - See 2.6.5) or renilla luciferase (for p65-GFP stimulation) were used as internal controls. Cells were co-transfected with pcDNA3.1 empty vector, MC160 or NPro as controls; or wild-type MGF360-12L.V5, or mutant variants MGF360-12L.mVHL.V5, MGF360-12L.mBC.V5, MGF360-12L.mCUL.V5; or wild-type MGF505-1R.V5 or mutant variants MGF505-1R.mSOCS.V5, MGF505-1R.mBC.V5 or MGF505-1R.mCUL.V5 (Figure 7-2). Further co-transfection of plasmids expressing constitutively active IRF3-5D or p65-GFP were used to stimulate the IRF3 and NF κ B pathways, respectively.

Using this system I was able to reveal that mutation of the novel SOCS-Box motif found in MGF505-1R significantly alleviated inhibition of stimulation of an NF κ B dependant luciferase reporter by exogenous expression of p65-GFP. It was clear that the introduction of mutations disrupting the full novel SOCS-Box (MGF505-1R.mSOCS.V5), the BC-Box region of the motif (MGF505-1R.mBC.V5), and the Cul5-Box region (MGF505-1R.mCUL.V5) reduced the inhibitory impact of MGF505-1R on the NF κ B pathway (Figure 7-5A). Interestingly mutation of the

whole motif had the greatest impact, whereas mutation of the BC-Box or Cul5-Box alone also significantly alleviated inhibition but to a much lesser effect, indicating that destruction of the entire motif is required for full abrogation of the inhibitory function. However, this was not the case for MGF360-12L. I observed that significant inhibition of the NF κ B pathway still occurred using mutations in the complete VHL-Box motif (MGF360-12L.mVHL.V5), BC-Box (MGF360-12L.mBC.V5) and Cul2-Box (MGF360-12L.mCUL.V5) (Figure 7-5A).

I investigated the impact of mutations in the MGF360-12L and MGF 505-1R proteins on inhibition of the IRF3 pathway following co-transfection of a plasmid expressing the constitutively active IRF3-5D. Mutation of the full SOCS-Box motif in MGF505-1R.mSOCS.V5 mutant reduced inhibitory activity suggesting the mutated sequence was critical for the inhibition (Figure 7-5B). In contrast the MGF505-1R.mBC.V5 and MGF505-1R.mCUL.V5 mutants retained inhibitory function of IRF3 luciferase suggesting that mutation of the BC-Box or Cul5-Box alone was not sufficient to alleviate the IRF3 inhibitory function of MGF505-1R (Figure 7-5B). Conversely MGF360-12L mutants (MGF360-12L.mVHL.V5, MGF360-12L.mBC.V5, and MGF360-12L.mCUL.V5) retained their inhibitory effect on the IRF3 pathway, with no significant difference observed between wild-type and mutant variants (Figure 7-5B). Thus, mutation of the VHL, BC or Cul2 boxes did not have an impact on the IRF3 inhibitory function of the MGF360-12L protein, and thus the motif is not responsible for the inhibitory mechanism.

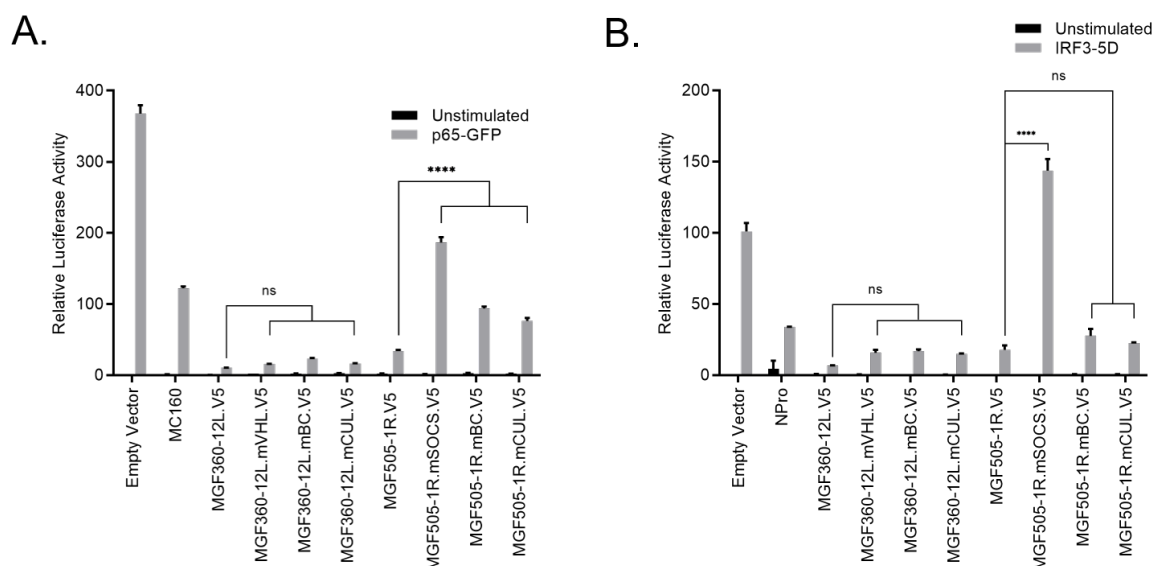


Figure 7-5. Mutations in the SOCS-Box of MGF505-1R alleviate inhibition of the IRF3 and NFkB pathways Relative luciferase assays investigating the impact of mutations in the putative VHL-Box of MGF360-12L and SOCS-Box of MGF505-1R on their (A) NFkB, and (B) IRF3 pathway inhibitory function. HEK293T cells were transfected with firefly luciferase reporter plasmids under the control of either (A) NFkB or (B) IRF3 response elements, and with control plasmids expressing renilla luciferase (A) or β gal (B). Cells were co-transfected with MGF360-12L or mutant variants (MGF360-12L.mVHL.V5, MGF360-12L.mBC.V5, MGF360-12L.mCUL.V5), MGF505-1R or its mutant variants (MGF505-1R.mVHL.V5, MGF505-1R.mBC.V5, MGF505-1R.mCUL.V5). Cells were also co-transfected with plasmids expressing MC160, or NPro as positive controls. Cells were co-transfected with plasmids expressing (A) p65.GFP or (B) IRF3-5D to stimulate NFkB or IRF3 driven luciferase reporter constructs, respectively. Data shown is representative of three experiments. For statistical analysis, two-way ANOVA Tukey's multiple comparison test was performed comparing stimulated wild-type MGFs to stimulated mutant MGFs. **** = $p \leq 0.0001$, *** = $p \leq 0.001$, ** = $p \leq 0.01$, * = $p \leq 0.05$.

7.2.5 MGF360-12L Reduced the Expression of Specific Transfected Plasmids

As previously described in 5.2.5, I observed a reduction in pRK5.flag.TRAF6 expression in the presence of MGF360-12L (Figure 5-6B) however on further inspection I did not see any change in endogenous expression of TRAF6 (Figure 5-7). I further investigated the effect of MGF360-12L on exogenous expression of proteins from other co-transfected plasmids (Figure 7-6) by western blotting. In these experiments I observed a severe reduction in expression from plasmids co-transfected with the MGF360-12L expressing plasmid including pCMV.p65.GFP, pCMV5.p300.HA, pEF.TBK1.flag, pcDNA3.1.Cul2.Myc, pcDNA3.1.Cul5.Myc, and pcDNA3.1.EloB.HA (Figure 7-6). Due to the number and variety of proteins affected it seemed unlikely that MGF360-12L is directly targeting all the proteins directly for degradation, whereas a reduction in transcription or translation would be a more plausible explanation. However, curiously this was not the case for luciferase endogenous control plasmids, pTK.renilla, pJAT.LacZ (Table 7-2). Furthermore, I did not observe alteration in the expression of endogenous proteins, including housekeeping genes such as α -Tubulin or β 3-Tubulin. Taken together this indicates that if indeed the reduction in expression from plasmids co-transfected with MGF360-12L is due to transcriptional or translational inhibition, it is specific and not broadly acting. Interestingly the CMV, or EF-1 α promoters that drive expression of pcDNA3.1, pRK5, and pCMV, or pEF plasmids share a common feature, since each contain NF κ B regulatory elements (Liu, Yuan *et al.* 2010, Zheng and Baum 2014, Brown, Sweeney *et al.* 2015, Wang, Xu *et al.* 2017). Conversely this is not the case for the herpes simplex virus thymidine

kinase (TK) promoter found in pTK.Renilla, or β -actin promoter of pJAT.LacZ (Masson, Ellis *et al.* 1992, Antonaki, Demetriades *et al.* 2011). In addition to this I also saw that the background level (unstimulated) of firefly luciferase expression when under the control of an NF κ B response element was also remarkably lower when MGF360-12L was concurrently expressed (Table 7-2). Thus, a plausible explanation is that the MGF360-12L protein inhibits transient expression driven by promoters containing NF κ B elements. This effect was nowhere near as obvious for firefly luciferase expression when controlled by IRF3 response elements (Table 7-2), potentially hinting towards separate mechanisms of action for MGF360-12L mediated inhibition of NF κ B and IRF3 pathways. However previous work has shown that the viral protein does not appear to impact upon the endogenous levels of protein thus not having a global impact (See endogenous proteins in Figure 5-5, Figure 6-4). Moreover, MGF360-12L clearly caused a reduction in exogenous expression of TRAF6 (Figure 5-6), but not with the endogenous protein (Figure 5-7). Further work is required to confirm the impact of MGF360-12L and identify the specific mechanism of inhibition of plasmid expression.

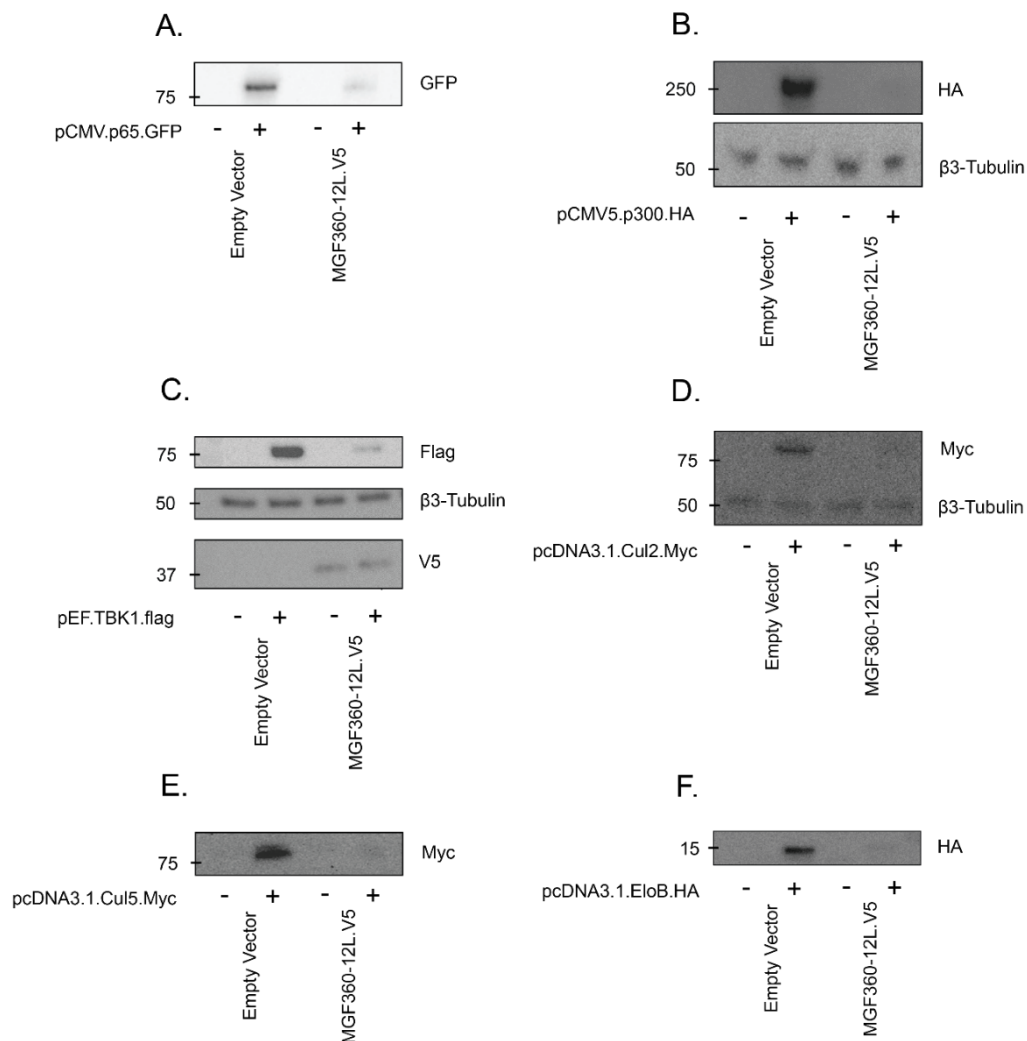


Figure 7-6. Impact of MGF360-12L on expression of exogenous plasmids

HEK293T cells were transfected with plasmids expressing empty vector control pcDNA3.1 or MGF360-12L.V5, and co-transfected with one of the following plasmids **A.** pCMV.p65.GFP, **B.** pCMV5.p300.HA, **C.** pEF.TBK1.Flag, **D.** pcDNA3.1.Cul2.Myc, **E.** pcDNA3.1.Cul5.Myc and, **F.** pcDNA3.1.EloB.HA. Cells were lysed and lysates were analysed by western blotting following SDS-PAGE separation. Exogenous protein expression was investigated by probing with antibodies targeting relevant protein tags. Molecular weight protein ladders were run in parallel and their position in kDa are noted on the left-hand side. β3-Tubulin was used as a loading control. A.C.D. are representative of experiments run in at least duplicate .

	Empty Vector		MGF360-12L.V5	
pTK.Renilla	11611	11400	13853	12936
p.LacZ	8454	9210	6223	6820
p.NFκB.luc	2614	1982	717	696
p.IRF3.Luc	55464	55091	51498	32108

Table 7-2 Typical values of unstimulated luciferase assay readings HEK293T cells were transfected with pcDNA3.1 empty vector as control or plasmids expressing MGF360-12L.V5. Cells were co-transfected with plasmids expressing internal controls pTK.Renilla, or p.LacZ, and firefly luciferase expressing plasmids under the control of the NFκB response elements (p.NFκB.luc) or IRF3 response elements (p.IRF3.Luc). Values are raw luciferase readings of technical replicates of one experiment. Values are representative of experiments performed in at least triplicate.

7.2.6 MGF505-1R's SOCS-Box Motif is Required for a Reduction in Endogenous Levels of p300

Here using western blot analysis I investigated the impact of MGF505-1R on endogenous levels of CBP and p300 cofactors. HEK293T cells were transfected with pcDNA3.1 empty vector as control, or wild-type MGF505-1R.V5, or mutant variants; MGF505-1R.mSOCS.V5, MGF505-1R.mBC.V5 or MGF505-1R.mCUL.V5 (Figure 7-2). Cells were lysed and analysed by western blotting. Using antibodies against endogenous p300, and CBP I observed that MGF505-1R significantly reduced levels of p300 but interestingly not the closely related paralog CBP (Figure 7-7A – Lane 2, Figure 7-7C). Importantly this reduction of p300 was not observed in cells transfected with the plasmid expressing the mutation of the full SOCS-Box motif MGF505-1R.mSOCS.V5 (Figure 7-7A – Lane 3, Figure 7-7C) and MGF505-1R.mCUL.V5 (Figure 7-7A – Lane 5). Mutation of the BC-Box alone appeared insufficient to fully rescue the effect on p300 expression (Figure 7-7A – Lane 4), thus potentially demonstrating that the Cul-Box motif alone is enough for functionality, however further work is required to fully determine this and quantify the effect. The reduction in amount of p300 would be explained by MGF505-1R targeting p300 for Ub mediated degradation as predicted from the presence of the SOCS-Box motif. This might involve a direct interaction between MGF505-1R and p300. However following transfection of a plasmid expressing MGF505-1R V5 into cells and CoIP using anti-V5 antibody I was unable to show an interaction between MGF505-1R and endogenous p300 (Figure 7-7B – Lanes 5 and 6). Similar experiments were carried out to investigate if transiently expressed MGF360-12L caused a reduction in the level of endogenous p300 or

CBP however expression of this protein did not appear to have any impact upon the level of either of these transcription cofactors (Figure 7-8).

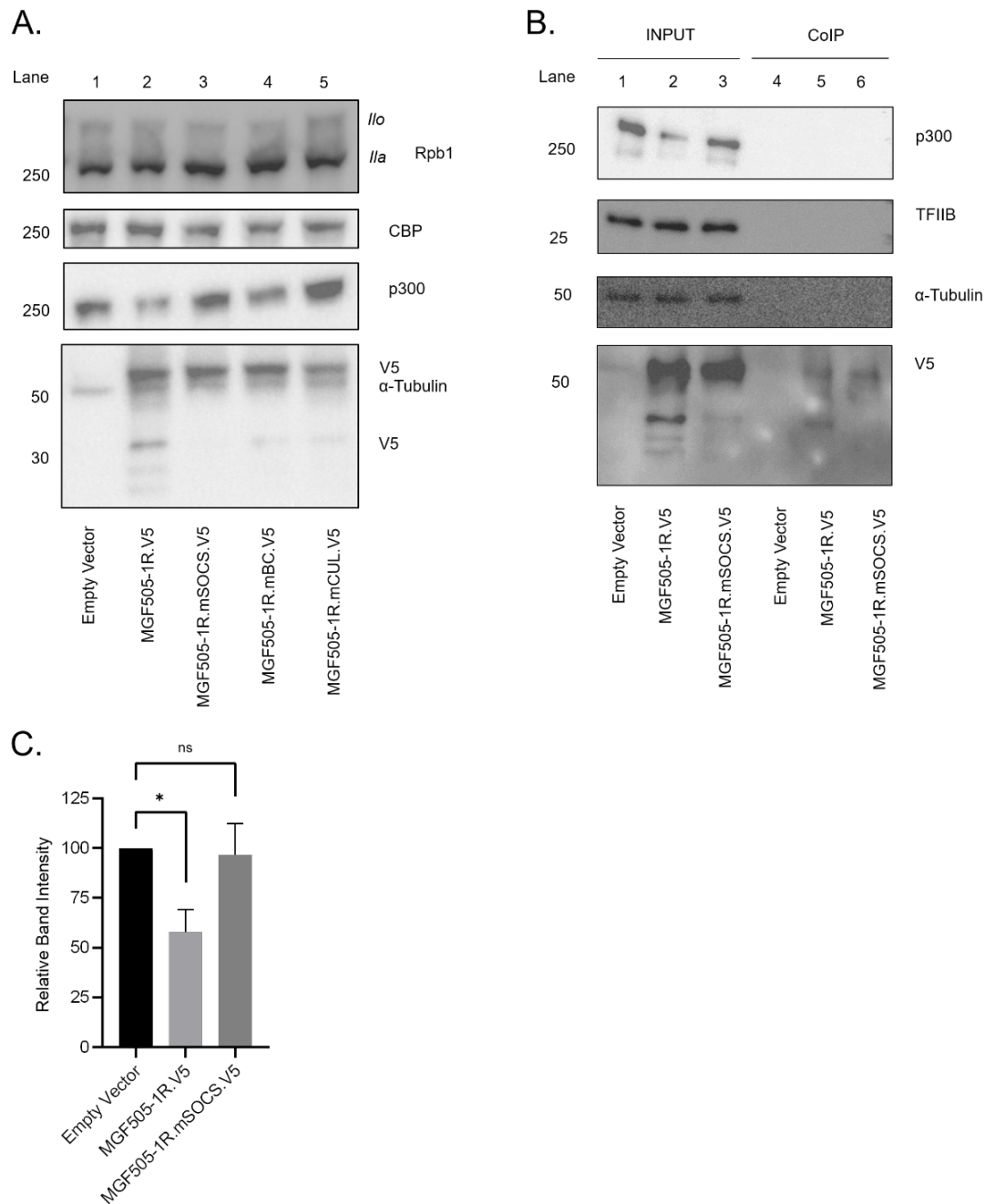


Figure 7-7. MGF505-1R impacts upon the level of endogenous p300 Western blot analysis investigating impact of MGF505-1R on the level of endogenous p300 co-activator and TFIIB. HEK293T cells were transfected with pcDNA3.1 empty vector as control, or wild-type MGF505-1R.V5 or mutant variants MGF505-1R.mSOCS.V5, MGF505-1R.mBC.V5, MGF505-1R.mCul.V5. Cells were lysed and lysates were investigated by western blotting following SDS-PAGE separation. Western blots

assessing Empty vector, MGF505-1R.V5, and MGF505-1R.mSOCS.V5 are representative of experiments performed in triplicate. **B.** CoIP to probe for interactions between MGF505-1R.V5, or MGF505-1R.mSOCS.V5 and endogenous p300 or TFIIIB. Lysates or CoIPs are shown from HEK293T cells transfected with empty vector (Lane 1 and 4) or, V5-tagged viral genes (MGF505-1R.V5 (Lane 2 and 5), MGF505-1R.mSOCS.V5 (Lane 3 and 6)). All cells were incubated in DSP cross linker prior to lysis. Sample of lysates were taken as "Input" (Lanes 1-3). The remaining lysate was incubated with magnetic beads covalently linked to anti-V5 antibodies to pulldown viral protein (CoIP - Lanes 4-6). Samples were analysed by western blotting following SDS-PAGE separation. Bands corresponding to V5-tag of MGF505-1R.V5 and MGF505-1R.mSOCS.V5 were observed in the Input (Lanes 2 and 3) and CoIP (Lanes 5 and 6) at the correct molecular weight. Input and CoIP were probed with antibodies against endogenous TFIIIB and p300. Molecular weight protein ladders were run in parallel and their position in kDa are noted on the left-hand side. α -Tubulin was used as endogenous loading control. Co-immunoprecipitation was performed in concurrence with CoIP in Figure 5-8 and Figure 7-4A. Therefore, α -Tubulin endogenous control and V5 probing is the same. **C.** Quantification using ImageJ software was performed to determine the relative change in band intensity of endogenous p300 between empty vector, MGF505-1R.V5 and MGF505-1R.mSOCS.V5. Tukey's test was performed for statistical analysis - * = $p < 0.05$.

7.2.7 Neither MGF360-12L or MGF505-1R Impacted upon TFIIB or Rpb1

Endogenous Protein Levels

RNAPII subunit Rpb1 is phosphorylated at 52 heptad repeats, and thus exists as hyperphosphorylated (Ilo) or hypophosphorylated (Ila) variants which are believed to influence transcriptional function (Phatnani and Greenleaf 2006, Chen, Smith *et al.* 2018). Using CoIP I investigated potential endogenous interactors of MGF360-12L and MGF505-1R. HEK293K cells were transfected with plasmids expressing wild-type MGF360-12L.V5 or MGF505-1R.V5, or mutant variants MGF360-12L.mVHL.V5, or MGF505-1R.mSOCS.V5, and incubated in DSP crosslinking agent. Input and co-immunoprecipitate were probed for the presence of Rpb1 using an antibody targeting both the hypophosphorylated (Ila) and hyperphosphorylated (Ilo) endogenous protein variants. I did not see any alterations in the level proteins in the presence of either MGF360-12L (Figure 7-8 – Lanes 2-3), or MGF505-1R (Figure 7-7B – Lanes 2 and 3), nor an interaction between Rpb1 and the viral proteins. Furthermore, a major component of the PIC and general transcription factor, TFIIB, has been demonstrated to be essential in RNAPII activity and interacts directly with transcription factors and co-activators to facilitate transcription (See 1.1.3) (Kim and Maniatis 1997, Kim, Kim *et al.* 1998). This was therefore an obvious area of investigation for interaction with MGF360-12L and MGF505-1R via CoIP. I was however unable to observe any clear and obvious impact upon the endogenous expression of TFIIB, and an interaction with the general transcription factor with MGF360-12L or MGF505-1R (Figure 7-8 and Figure 7-7B).

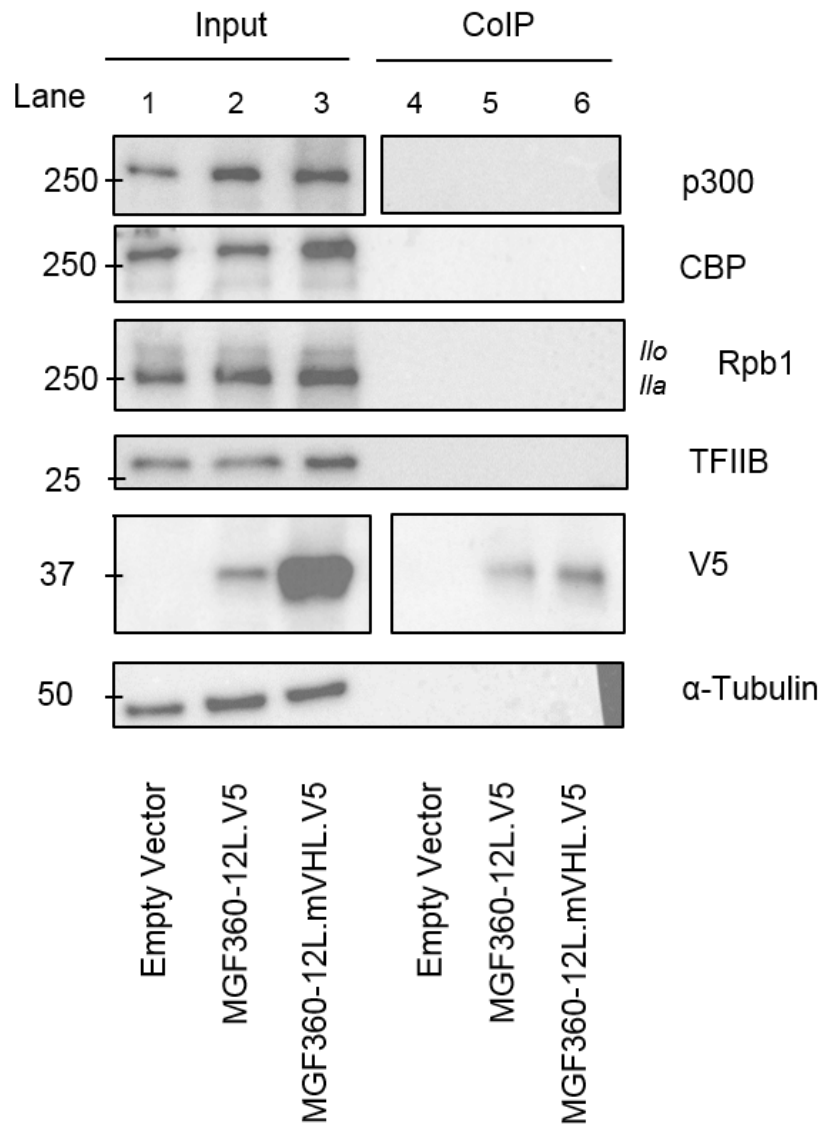


Figure 7-8. CoIP probing for interacting partners of MGF360-12L HEK293T cells were transfected with empty vector pcDNA3.1 as control, or MGF360-12L.V5 or mutant variant MGF360-12L.mVHL.V5. Cells were incubated in DSP crosslinker prior to lysis. Sample of lysate was taken as **Input** (Lanes 1-3). For **CoIP**, the remaining lysates were incubated in magnetic beads covalently linked to anti-V5 antibodies with the aim of pulling down V5-tagged viral proteins and any interacting partners (Lanes 4-6). Input and CoIP were analysed by western blotting following SDS-PAGE separation and probed using antibodies targeting V5-tagged viral proteins, or endogenous proteins p300, CBP and Rpb1. Input showed banding

for p300, CBP and Rpb1 at the correct molecular weight (Lanes 1-3), V5 banding was also seen at the correct molecular weight (Lanes 2 and 3). Immunoprecipitation of MGF360-12L.V5 (Lane 5), or MGF360-12L.mVHL.V5 (Lane 6), showed banding at correctly molecular weight when probed with anti-V5 antibodies. Bands corresponding to proteins p300, CBP and Rpb1 were not observed in CoIP (Lanes 5 and 6). Molecular weight protein ladders were run in parallel and their position in kDa are noted on the left-hand side. α -Tubulin was used as a loading control. The CoIP assay was performed in concurrence with CoIP in Figure 5-7, Figure 6-5, and Figure 7-3A. Therefore α -Tubulin endogenous control, and V5 probing is the same.

7.3 Discussion

Here I determined that despite similarities the putative VHL/SOCS-Box-like motifs found at the N-terminus of MGF360-12L and MGF505-1R are functionally distinct (Table 7-3). The motif found in MGF360-12L functionally resembles a VHL-Box, whereas the MGF505-1R motif resembles a SOCS-Box (Table 7-1). Using CoIP, I showed that MGF360-12L interacted with endogenous EloB, EloC and Cul2 but importantly not Cul5, therefore strongly suggesting that the motif is a VHL-Box, rather than a SOCS-Box which utilises Cul5 (Figure 7-3 and Figure 7-4A). However, this was not the case for MGF505-1R. Despite being able to co-immunoprecipitate endogenous EloB, I was unable to do so with exogenous EloC (Figure 7-4D). I was however able to successfully pulldown exogenously expressed Cul5 (Figure 7-4C), whereas this was not possible with exogenous Cul2 (Figure 7-4B). This therefore demonstrates that unlike MGF360-12L, the motif found in MGF505-1R has functional characteristics of a SOCS-Box, a surprising finding considering the sequence of the motif found was more reminiscent of a VHL-Box (See 3.2.4). Interestingly SOCS-Box protein Elongin A (EloA), despite showing a similarly degraded motif lacking the LPΦP sequence, thought to be essential in binding Cul5 and thus more reminiscent of a Cul2-Box, retains its Cul5 binding capacity (Mahrou, Redwine *et al.* 2008). Further weight is added to the hypothesis that the putative VHL/SOCS-Box motifs are responsible for these interactions as all interactions were absent (or greatly reduced in the case of MGF505-1R - Cul5) on the mutation of the full-length motif. However, MGF505-1R.mSOCS appeared to interact with Cul5, since a very weak band relative to the wild type protein was observed by CoIP in cells exogenously

expressing Cul5. This could be explained by the mutation not fully abrogating the interaction and residual binding is observed, and may have been further compounded by exogenously expressing Cul5 not being physiologically representative and essentially forcing the interaction. Further work is required to assess the functionality of the individual BC-Box and Cul2/5-Boxes of the motif. Moreover, the lack of an interaction with the mutant variants demonstrates that the interaction is not due to the presence of DSP crosslinking reagent used to treat cells. However, as the use of the crosslinking agent was necessary to observe the interaction may indicate that the interaction is short-lived or weak.

MGF	Interacting partners	Inhibition of the NFκB pathway?	Inhibition of the IRF3 pathway?	Reduction of endogenous p300 levels?
MGF360-12L	Endogenous EloB, EloC, Cul2	Yes	Yes	No
MGF360-12L.mVHL	Not observed	Yes	Yes	No
MGF360-12L.mBC	Not tested	Yes	Yes	Not tested
MGF360-12L.mCUL	Not tested	Yes	Yes	Not tested
MGF505-1R	Endogenous EloB Exogenous Cul5	Yes	Yes	Yes
MGF505-1R.mSOCS	Potential weak interaction with exogenous Cul5	Significantly reduced	Significantly reduced	No
MGF505-1R.mBC	Not tested	Significantly reduced – Less so than MGF505-1R.mSOCS	Yes	Potentially
MGF505-1R.mCUL	Not tested	Significantly reduced – Less so than MGF505-1R.mSOCS	Yes	No

Table 7-3. Overview of results investigating role of the VHL/SOCS box in the functionality of MGF360-12L and MGF505-1R.

As I had previously demonstrated an inhibitory impact of MGF360-12L and MGF505-1R on both the NFκB and IRF3 pathways, to assess the functional

impact of the VHL-Box or SOCS-Box motifs, NFκB and IRF3 dependant luciferase assays were performed with the mutant variants (Figure 7-2 and Figure 7-5). Importantly, mutation of the full motif in MGF505-1R clearly demonstrated a significant reduction of inhibition of the NFκB pathway and a full reduction of IRF3 pathway. Mutation of the full SOCS-Box motif and individual BC-Box and Cul5-Box of MGF505-1R saw a significant alleviation of inhibition relative to wild-type in the NFκB pathway, however this inhibition broadly remained but to a much lesser extent. Whereas inhibition of the IRF3 pathway was fully relieved with the full SOCS-Box motif mutations, whereas it was retained regardless of mutations of the individual components of the SOCS-Box. Alas, this was not the case for MGF360-12L. Despite the protein interacting with endogenous proteins of CRL machinery, mutation of the full length VHL-Box motif, or constituent parts (BC-Box and Cul2-Box) did not alleviate the inhibitory function of the protein. Therefore the motif may perform an additional, as of yet unknown, role in the functionality of MGF360-12L. As the motif is recruiting the CRL machinery, it appears to fully operational, therefore such unknown functionality may include targeting proteins outside of the IFN pathways assayed for ubiquitination. However, whether the recruitment of EloB, EloC and Cul2 is sufficient for activity of the CRL remains to be seen. Furthermore, the requirement of the crosslinking agent may indicate that the interaction between the viral proteins and the CRL machinery may be transient or weak, and therefore may not be physiologically representative. Alternatively, the pulldown of the V5 tagged viral proteins was weak in some instances, thus insufficient viral protein may have been present to coimmunoprecipitate with sufficient cellular proteins. Additional work is required to further clarify the results.

Numerous viral proteins have been shown to modulate the CRL machinery. Epstein Barr virus BZLF1 protein contains a SOCS-Box and is capable of recruiting the CRL targeting p53 for degradation (Sato, Kamura *et al.* 2009). Similarly HPV16 E7 recruits Cul2 based CRL via an adaptor protein Zer1 targeting retinoblastoma (White, Sowa *et al.* 2012). As previously described in (See 1.1.7.4) a wide range of poxvirus ANK proteins deploy the highly similar F-Box motif recruiting Skp1-Cul1 based CRL machinery (Mercer, Fleming *et al.* 2005, Sonnberg, Seet *et al.* 2008, Mohamed, Rahman *et al.* 2009, Sonnberg, Fleming *et al.* 2009, Burles, van Buuren *et al.* 2014). Many of these poxvirus ANK proteins are known to target the IFN response, however the specific targets of these proteins for ubiquitination generally remains elusive (Mohamed, Rahman *et al.* 2009, Burles, van Buuren *et al.* 2014).

In addition to the poxvirus PRANC motif proteins that recruit CRL machinery via an F-Box (See 1.1.7.4), poxvirus proteins which utilise the CRL machinery in a VHL-Box or SOCS-Box manner have been reported, albeit much less frequently. Odon, Georgana *et al.* (2018) reported that a novel class of chordopoxvirus ANK repeat proteins recruited Cul2 CRL machinery via a BC-Box (although they did not describe a Cul2-Box). Of the family, Ectromelia virus 010 protein demonstrated clear inhibition of the NF κ B and IRF3 pathways at a step downstream of stimulation of multiple PRRs, and moreover suppressed the expression of IFN, and CXCL10 and CCL5 cytokines after Sendai virus stimulation. Intriguingly, it was shown by mutagenesis of the BC-Box motif that the recruitment of the CRL was

essential to the protein's inhibitory function of NF κ B but not IRF3. However, the authors were unable to demonstrate the protein's specific substrate target (Odon, Georgana *et al.* 2018). Poxvirus proteins recruiting Cul5 based CRL complexes have also been reported to impact upon the IFN pathways. MC132 from molluscum contagiosum virus recruits EloB and EloC via a complete BC-Box, and Cul5 via a partial Cul5-Box, in order to target p65 for degradation (Brady, Haas *et al.* 2015). By demonstrating in this chapter that both MGF360-12L and MGF505-1R are present in Cul2 or Cul5 CRL complexes respectively, and that for MGF505-1R at least the motif is essential for the inhibition of NF κ B and IRF3 pathways, it would not be beyond reasonable doubt to suggest that the mechanism of inhibition of the ASFV proteins would be by using the CRL to target cellular proteins for destruction. I therefore began to investigate potential interacting cellular partners.

The data collected thus far has indicated that MGF360-12L and MGF505-1R are capable of strongly inhibiting both the NF κ B (Chapter 5) and IRF3 (Chapter 6) pathways, but also appear to retain the functionality of the pathway itself since they do not block nuclear translocation (with the exception of MGF360-12L which partially limits IRF3 nuclear translocation) nor interacted with the transcription factors directly. It was therefore a logical step to begin to probe common components post-nuclear translocation of NF κ B and IRF3. Moreover, I showed that MGF360-12L selectively inhibits expression of exogenous proteins potentially dependent upon the promoter driving expression. This possibly further hints towards the MGF360-12L protein inhibiting transcription or translation

(Figure 7-6 and Table 7-2). Curiously, this was not the case with endogenous proteins including housekeeping genes, and thus a broad mechanism of action can be ruled out. The data presented in this chapter show the presence of an NFκB element in the promoters driving expression may determine levels of exogenous protein expression. Thus, an effect of MGF360-12L on transcription driven from the promoter is a possible explanation for the results, however significantly more work is required to further examine this and confirm the impact of on transcription. Transcriptome analysis by RNAseq may offer insight to whether MGF360-12L is preferentially inhibiting transcription of specific genes.

I investigated the impact of MGF360-12L and MGF505-1R expression on levels of transcription cofactors CBP and paralog p300, both essential in the induction of gene expression under the control of NFκB or IRF3 regulatory sites (Gerritsen, Williams *et al.* 1997, Merika, Williams *et al.* 1998, Yoneyama, Suhara *et al.* 1998, Mukherjee, Behar *et al.* 2013). Here I did not observe any change to the level of CBP with expression of either MGF360-12L, or MGF505-1R. However importantly MGF505-1R induced a significant reduction in endogenous p300 level, which was rescued with the mutation of the full SOCS-Box (Figure 7-7A and C). I was unable to show an interaction between the p300 and MGF505-1R proteins however (Figure 7-7B). It was interesting that despite seeing a reduction in endogenous p300 levels following MGF505-1R expression I did not see any generalised inhibition of housekeeping gene expression nor inhibition of the Gal4 luciferase assay systems (Figure 5-9 and Figure 6-6). CBP and p300 share extensive homology and overlapping features, and are generally considered functionally

identical, therefore the specific targeting of p300 but not CBP by MGF505-1R is curious. There is however some evidence that the cofactors may perform distinct functions (Kung, Rebel *et al.* 2000, Chan and La Thangue 2001, Fauquier, Azzag *et al.* 2018). *In vivo* knockout models for example have shown that p300 or CBP heterozygous null mice display different phenotypes (Kung, Rebel *et al.* 2000). Furthermore, Fauquier, Azzag *et al.* (2018) demonstrated that the co-activators complement each other by regulating a unique gene profile in human myoblast differentiation. Both cofactors are thought to play an identical role in IFN transcription, and the importance of these described differences to this specific pathway is not known. As to why one cofactor is targeted and the other is not; the simplest explanation may be that targeting p300 alone is sufficient to severely limit the IFN response. Despite the shared domain architecture and sequence similarity of roughly 70%, the proteins share much lower homology outside functional domain regions (Dancy and Cole 2015), if MGF505-1R were to hypothetically target p300 via these regions this would offer a possible explanation for not observing an impact on CBP. Deeper examination whether MGF505-1R is interacting with p300 is needed. Exogenous expression of the cofactor in the CoIP assay, or alternate procedures such as *in situ* proximity ligation assay may offer more insight.

As CBP/p300 have been shown to play a crucial role in the formation of the enhanceosome to induce an appropriate immune response, it is therefore not surprising that they are a target for viral proteins (See 1.1.3). In fact, viral targeting of these co-activators has been previously reported. Both human

Herpes simplex virus 1 (HSV1) and bovine herpes virus Zinc-RING finger protein ICP0 inhibit IRF3 driven IFN expression by recruiting activated IRF3 and CBP/p300 (Zhang, Jiang *et al.* 2006, Melroe, Silva *et al.* 2007). CoIP revealed that transfected bovine ICP0 interacts with p300 on its exogenous expression, whereas interaction with the endogenous protein was considered too difficult due to insufficient protein levels (Zhang, Jiang *et al.* 2006). Interestingly Zhang, Jiang *et al.* (2006) showed that ICP0 utilised p300 to enhance viral promoter activity and proposed that the interaction indirectly prevented histone acetylation and induction of expression of IFN genes. Adenovirus oncoprotein E1A interacts with p300 and retinoblastoma to control transcription of a wide array of host genes but shows a striking repression of ISG under control of ISRE sites specifically (Gutch and Reich 1991, Ferrari, Gou *et al.* 2014, Zemke and Berk 2017). Interestingly, like MGF505-1R, the cellular C6orf106 protein was shown to be required for henipavirus replication by blocking IRF3 and NFκB pathways but does not inhibit pathway viability above the level of transcription factor nuclear translocation. Furthermore, C6orf106 reduced p300 and CBP levels preventing IRF3 association with the promoter and thus impairing transcriptional transactivation (Ambrose, Liu *et al.* 2018). Porcine epidemic diarrhoea virus Nsp1 degrades p300 paralog CBP in a proteasomal manner effectively interrupting the IRF3 enhanceosome formation. However, Nsp1 did not interact with either IRF3 nor CBP (Zhang, Shi *et al.* 2016). Finally, ASFV protein A238L has been shown to inhibit the expression of cyclooxygenase-2 (COX-2) and TNFα by preventing coactivator phosphorylation and activation thus displacing CBP/p300 from the promoter (Granja, Nogal *et al.* 2006, Granja, Sánchez *et al.* 2009). Together this would imply that prevention of p300 induced transcription is an inhibition method

capable of targeting IFN gene expression with little impact on general cellular transcription. This may explain why MGF505-1R can repress transcription of NF κ B or IRF3 driven genes but is not broadly acting.

Intriguingly, elongins have been shown to regulate transcription. EloB and EloC heterodimer has been found to interact with the transcriptionally active EloA, an RNAPII elongation factor and SOCS-Box protein (Aso, Haque *et al.* 1996). Together this heterotrimer is known as Elongin (SIII). Under normal cellular conditions the complex interacts with RNAPII, strongly inducing transcription via reducing transient pausing of the transcription machinery to encourage more efficient RNA alignment with the polymerase enzyme (Aso, Lane *et al.* 1995, Aso, Haque *et al.* 1996, Yasukawa, Sugimura *et al.* 2007). EloA and EloC together appear to be important for the transcriptional activity, whereas EloB acts as a chaperone for assembly and is not required for transcriptional activity (Aso, Lane *et al.* 1995). Furthermore, VHL tumour suppressor protein, from which the VHL-Box derives its name, competes with Elongin (SIII) by sequestering EloB and EloC to prevent activation of EloA and thus transcriptional activity (Duan, Pause *et al.* 1995, Aso, Haque *et al.* 1996). Conversely in conditions of ultraviolet irradiation induced cellular stress, the Elongin (SIII) complex induces ubiquitination and degradation of Rpb1 via further Cul5 recruitment and formation of CRL E3 ligase (Yasukawa, Kamura *et al.* 2008). Viral interference of Elongins impacting upon the IFN response has also been reported. For example, La Crosse Orthobunyavirus has been demonstrated to antagonise the IFN pathway by the viral protein NSs rapidly inhibiting transcription (Schoen, Lau *et al.* 2020). The

authors were unable to observe an interaction between NSs and EloC, but the viral protein caused an indirect re-localisation of the cellular protein, and was responsible for the resulting Rpb1 degradation and shutdown of IFN expression (Schoen, Lau *et al.* 2020). Considering this, I investigated whether MGF360-12L and MGF505-1R have any impact upon Rpb1. I was unable to observe any impact upon the protein level or any changes in hyperphosphorylated (Ilo) or hypophosphorylated (IIa) variants with either viral protein (Figure 7-8 and Figure 7-7). This therefore indicates the mechanism of action of the MGF360-12L or MGF505-1R is unlikely to be dependent upon alterations of Rpb1. However, investigating whether MGF360-12L or MGF505-1R are acting in a similar manner to VHL and preventing Elongin (SIII) complex formation would be of interest. If this were the case, hypothetically the exogenous expression of EloC would rescue inhibition of the NFκB/IRF3 driven luciferase assays.

Two modes of transcription activation are thought to exist, unpausing of RNAPII already positioned at promoters, or a TFIIB dependant *de novo* recruitment of the polymerase to transcriptional start sites (Kim, Kim *et al.* 1998, Haas, Meiler *et al.* 2018). IRF3 driven expression appears dependent on *de novo* recruitment whereas NFκB targets are regulated by both (Freaney, Kim *et al.* 2013, Haas, Meiler *et al.* 2018). The general transcription factor TFIIB recruits RNAPII to the promoter and is considered essential for transcription (Kim, Kim *et al.* 1998, Haas, Meiler *et al.* 2018). As TFIIB is a major component essential for the formation of the PIC and is thought essential for transcription of all genes, it would therefore seem logical that any targeting of the general transcription factor

would result in broad spectrum transcription shutoff. Curiously however this does not appear to be the case. Targeting TFIIB has been implicated in specific antagonism of the IFN pathway by limiting *de novo* recruitment of RNAPII to transcriptional start sites following pathway stimulation, but may not actually be required for release of paused polymerases (Vogt, Preuss *et al.* 2008, Gelev, Zabolotny *et al.* 2014, Haas, Meiler *et al.* 2018). Tick-borne orthomyxovirus Thogoto virus ML protein has been demonstrated to be a potent inhibitor of both the NF κ B and IRF3 pathways, reminiscent of MGF360-12L and MGF505-1R (Jennings, Martínez-Sobrido *et al.* 2005, Vogt, Preuss *et al.* 2008, Haas, Meiler *et al.* 2018). Moreover, ML has been shown to inhibit IRF3 dimerisation and association with CBP whilst allowing nuclear translocation, but does not directly interact with the transcription factor (Jennings, Martínez-Sobrido *et al.* 2005). However Vogt, Preuss *et al.* (2008) demonstrated that ML interacts with TFIIB, and proposed that this interaction may be acting as a platform to prevent CBP-IRF3 interaction. Haas, Meiler *et al.* (2018) further built upon this, revealing that ML does not degrade TFIIB but in fact re-localises it from the nucleus to the cytoplasm, creating a TFIIB depleted environment thus preventing *de novo* recruitment of RNAPII to the IFN promoter. Using siRNA knockdown of TFIIB and stimulating Hela cells with TNF α followed by RNA-seq global transcriptome analysis, they unexpectedly showed that TFIIB depletion preferentially affected antiviral and inflammatory gene expression but did not impact broadly on cellular transcription. Furthermore they showed that ML repressed a number of plasmids containing NF κ B sites, similar to my observations with MGF360-12L, suggesting promoters that rely on *de novo* recruitment of RNAPII, including IRF3 driven promoters, are directly impacted by ML (Haas, Meiler *et al.* 2018). I noted that

the MGF360-12L induced a reduction in expression of specific plasmids under the control of NFκB regulatory elements, but not others. Interestingly the CMV promoter found in many of the plasmids described contains NFκB binding sites important for transcription, but also SP1, CBP and tata-binding protein binding sites (Hogel, Laprairie *et al.* 2012). Despite not seeing an interaction between MGF360-12L and TFIIB, the similarities in action to Thogoto virus ML protein mean that further examination is needed. For example, epidermal growth factor driven gene expression does not appear to be dependent on *de novo* recruitment of TFIIB, further luciferase assays probing this pathway would warrant investigation (Haas, Meiler *et al.* 2018).

Here I have demonstrated that the previously identified VHL-Box and SOCS-Box of MGF360-12L and MGF505-1R respectively are functional in recruiting the CRL machinery. But importantly in the case of MGF505-1R the SOCS-Box plays a role in the inhibition of NFκB and IRF3 pathways. I propose that the mechanism of MGF505-1R is recruitment of p300 to the ANK repeat motif region of the protein, and additionally uses the recruitment of the CRL machinery via the N-terminus SOCS-Box motif, to target the cofactor for degradation. This in turn impairs expression via IRF3 and NFκB driven promoters. However, this does not explain why I did not observe inhibition of the Gal4 luciferase assays (Figure 5-9 and Figure 6-6). Despite the exact mechanistic action of inhibition for both proteins remaining elusive, the data thus far points towards a direct impact on transcription for both MGF360-12L and MGF505-1R rather than exclusively targeting upstream inhibition of the signalling pathways.

8 DELETION OF MGF360-12L, 13L, 14L, MGF505-1R & K145R FROM VIRULENT GEORGIA07/1 ALTERS ISG TRANSCRIPT LEVELS AND CONFERS PROTECTION AGAINST CHALLENGE WITH PARENTAL VIRUS

8.1 Introduction

ASFV is a highly lethal virus that threatens socio-economic growth and stability, and food security of regions of the globe that rely on pork as a critical source of protein and income (See 1.2). There is currently no vaccine available, leaving aggressive containment measures the only method of dealing with the virus, therefore, the urgent need for a vaccine is extremely evident.

Building upon the discovery that OURT88/3 provides limited protection as a vaccine, a range of studies were performed implicating that the deletion of various profiles of these 8 MGF genes attenuates ASFV of a number of isolates and genotypes (including Georgia07/1 the virus currently circulating in Europe and Asia), and provided some level protection on challenge, including up to 100% with genotype I (Boinas, Hutchings *et al.* 2004, Burrage, Lu *et al.* 2004, Abrams, Goatley *et al.* 2013, O'Donnell, Holinka *et al.* 2015, Golding, Goatley *et al.* 2016, Reis, Abrams *et al.* 2016). Despite these live attenuated viruses having shown some promise as vaccine candidates, many did not offer sufficient protection on challenge, or animals presented with clinical symptoms post vaccination (Burrage, Lu *et al.* 2004, O'Donnell, Holinka *et al.* 2015, Golding, Goatley *et al.*

2016). Importantly deletion of MGF360-12L, 13L, 14L, MGF505-1R, 2R, and 3R, has been shown to confer protection against wild-type genotype II viruses, however animals presented with low level febrile disease and viraemia of challenge virus (O'Donnell, Holinka *et al.* 2015, Chen, Zhao *et al.* 2020). Although previous work has revealed that large scale deletion of these genes is sufficient to attenuate the virus (See 1.2.4 and 1.2.6.3), little work has been performed to begin to understand which of the genes is actually responsible for this attenuation. In this thesis I attempted to understand the functional mechanisms that the MGFs deploy to subvert the IFN response. This was with the overarching aim of applying this knowledge to enable a rational selection of genes for deletion in order to begin to understand which genes are indispensable for the attenuation of genotype II Georgia07/1 and create a more appropriate LAV candidate.

Previous work on ASF vaccine development has struggled to show clear correlates of protection. However, some evidence has suggested a role for both antibodies, and cytotoxic CD8⁺ T cells. The role of antibodies continues to be debated; passive administration (Onisk, Borca *et al.* 1994), or maternal transfer via colostrum (Schlafer, Mebus *et al.* 1984) have both been shown to offer protection, although the specific mechanisms of which remain unclear. Moreover, using sera from pigs immunised with baculovirus expressed CD2v surviving challenge with virulent E75, Ruiz-Gonzalvo, Rodríguez *et al.* (1996) suggested antibodies were responsible for the inhibition of hemadsorption *in vitro*. However neutralising antibodies preventing virus internalisation and binding have also been proposed to play a role in protection (Escribano, Galindo

et al. 2013). Additionally, the importance of an appropriate antibody response is demonstrated by the suggestion that antibody dependant enhancement may be a factor to consider as a number of subunit vaccines resulted in heightened pathology (Blome, Gabriel et al. 2014, Netherton, Goatley et al. 2019, Sunwoo, Pérez-Núñez et al. 2019). The role of T cells has also been investigated but conclusive results are still unclear. Oura, Denyer et al. (2005) clearly demonstrated a role for T cells in ASF protection by showing that the depletion of CD8 α ⁺ cells, consisting of $\alpha\beta$ T cells but also $\gamma\delta$ T cells, NK cells and invariant T cells, abrogates protection. Likewise, CD4⁺CD8 $\alpha\beta$ ⁺ double positive T cells also function as cytotoxic T cells in swine and were shown to be significantly increased in pigs immunised with OURT88/3 and surviving challenge with OURT88/1, whereas conventional CD8⁺ single positive T cells were associated with the onset of symptoms (Denyer, Wileman et al. 2006). Interestingly, increased presence of regulatory T cells have been proposed to correlate with the absence of long term protection in pigs immunised with attenuated isolates (Sánchez-Cordón Pedro, Jabbar et al.). Overall, despite the specific mechanisms and profiles of humoral and cell mediated immunity in protection against ASF remaining vague, it is likely that both are needed to be induced by a vaccine for sterile immunity. Therefore, LAV candidates are generally thought to be the best placed to induce a robust immune response.

Here I demonstrate that the selection of MGF360-12L, 13L, 14L and MGF505-1R for deletion from a Georgia07/1 background was sufficient to induce a high level of ISG expression at early time points in *in vitro* infections, and importantly

attenuate the virus conferring protection to pigs challenged with the parental isolate. In contrast deletion of MGF360-13L, 14L and MGF505-2R and 3R did not achieve the same results. Due to the overlap in profiles, this therefore revealed that attenuation is likely dependent on MGF360-12L or MGF505-1R or both. This body of work paves the way for further research to isolate the specific gene or genes responsible for attenuation.

8.2 Results

8.2.1 Selection of a Profile of MGFs for Deletion from Georgia07/1

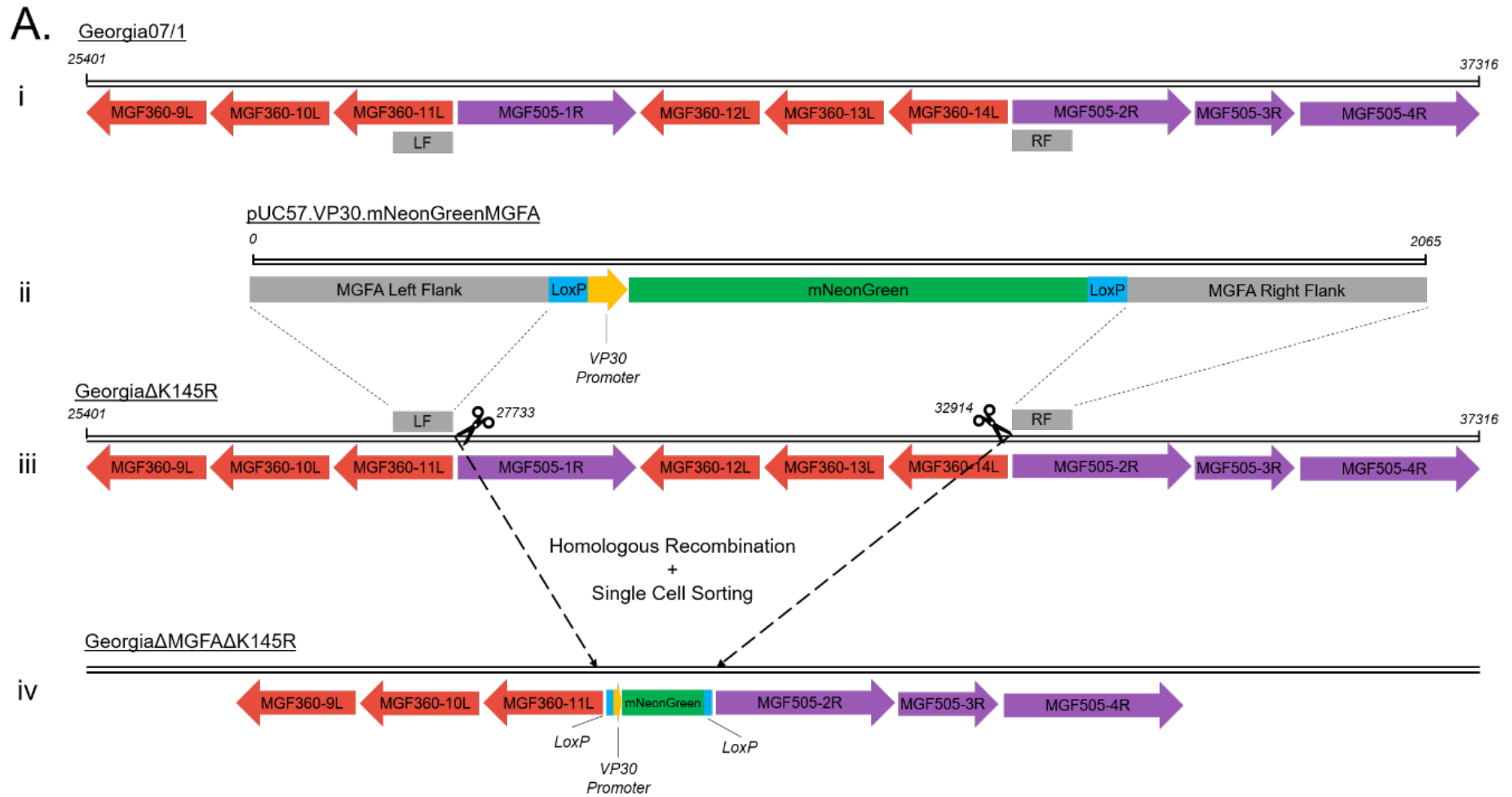
I selected two profiles of MGFs for deletion from Georgia Δ K145R. Georgia Δ K145R is a previously generated deletion mutant of genotype II parental virus Georgia07/1, lacking K145R nonessential gene, with the aim of the deletion being used as a differentiation of immunised and vaccinated animals (DIVA) target (data not shown). The criteria and rationale for selecting genes for deletion was three-fold. Firstly, the deletion should cover at least 1 gene from either MGF360 cluster (MGF360-9L, 10L, 11L, 12L, 13L and 14L) and MGF505 cluster (MGF505-1R, 2R and 3R), the profile of genes when deleted in unison shown to be responsible for attenuation (O'Donnell, Holinka *et al.* 2015, Reis, Abrams *et al.* 2016, Chen, Zhao *et al.* 2020). Secondly for simplicity the deletion must be achievable with one single homologous recombination event. Finally the data shown in Chapters 5, 6 and 7 investigating the functional mechanisms of the genes was used as the basis for rational selection. As all genes within contention for deletion appear to function as potent immunomodulatory genes rationally

selecting individual genes for deletion on this basis was difficult, however as MGF360-12L showed a highly potent inhibition of the IRF3 and NFκB pathways in a multifunctional manner that seemed unique, therefore this gene was considered an attractive target. As the data has shown that MGF505-1R was likely acting in a mechanism different to MGF360-12L, this gene was also chosen as a target in aim of securing a deletion that covered genes with at least 2 different functional mechanisms. Genome architecture played a major role in my decision for candidates for deletion, as a single deletion would be desirable. The region of MGF360-8L-14L is intersected by MGF505-1R flanked by MGF360-11L and 12L. The MGF505 clusters then continues after MGF360-12L with MGF505-2R-10R (Note MGF505-9R is absent in Georgia07/1) (Chapman, Tcherepanov *et al.* 2008, Chapman, Darby *et al.* 2011). Therefore, based upon this, two profiles of genes were selected for deletion. Deletion profile 1 included MGF360-12L, 13L, 14L and MGF505-1R which was termed MGFA. As the data has shown that MGF505-1R was likely acting in an alternate mechanism to that of MGF360-12L, this gene was chosen as a complimentary target in aim of securing a deletion that covered genes with at least two different functional mechanisms. The second profile of genes however included MGF360-13L, 14L and MGF505-2R and 3R termed MGFB.

Homologous recombination and single cell sorting were used to delete the selected genes to generate live attenuated deletion mutant vaccine candidates by Ana Reis and Anusyah Rathakrishnan. To facilitate homologous recombination, reporter cassette plasmid constructs were designed and

synthesized. pUC57 plasmid vectors were designed to contain the sequence of mNeonGreen under the control VP30 ASFV viral promoter, flanked by loxP region and 500 base pair of the flanking regions directly at either end of the areas of Georgia07/1 targeted for deletion (Figure 8-1 Aii and Bii). mNeonGreen was included as functional reporter to allow the selection of cells simultaneously transfected and infected. 2 constructs were designed corresponding to MGFA and MGFB deletion, named pUC57.VP30mNeonGreenMGFA and pUC57.VP30mNeonGreenMGFB respectively. ASFV VP30 promoter gene was chosen as a promoter due to having been shown to be expressed at early and late stages of infection and capable of driving strong expression, therefore mNeonGreen would be expressed during the whole cycle of infection (Portugal, Bauer *et al.* 2017). LoxP sites were included to facilitate removal of reporter genes using cre recombinase if needed (Abrams and Dixon 2012).

The process of generating deletion mutants was performed by Ana Reis and Anusyah Rathakrishnan, with the general protocol as described by (Rathakrishnan, Moffat *et al.* 2020).



B.

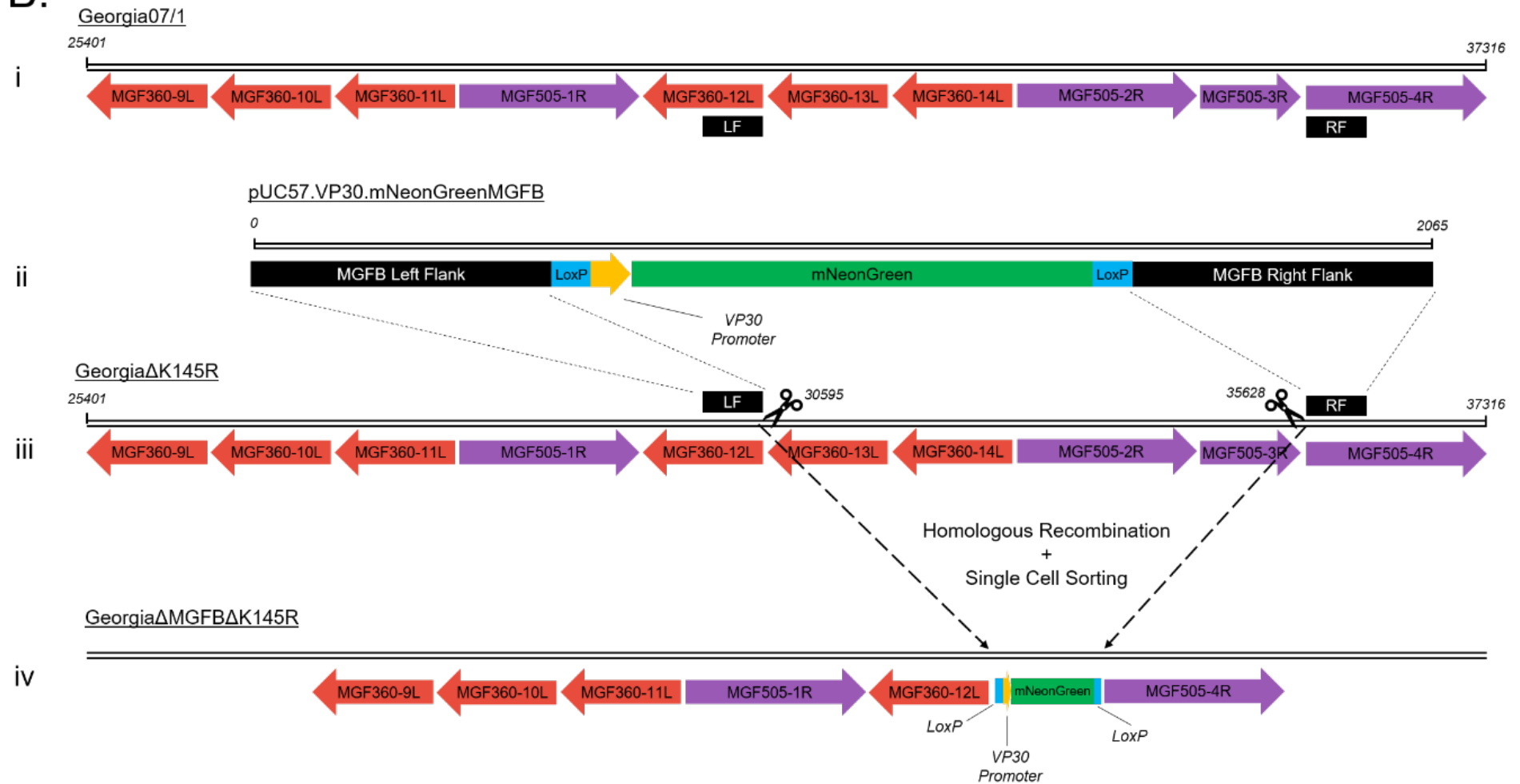


Figure 8-1. Schematic depicting the generation of deletion mutants lacking member of MGF360 and MGF505 genes **A.** Shows deletion of MGF360-12L, 13L, 14L and MGF505-1R from GeorgiaΔK145R, generating GeorgiaΔMGFAΔK145R. **B.** Shows deletion of MGF360-13L, 14L, MGF505-2R and 3R from GeorgiaΔK145R, generating GeorgiaΔMGFBΔK145R. **ABi.** Shows left terminal repeat genome of Georgia07/1 encompassing MGF360 and MGF505 of interest from genome position 25401 to 37316. Left flank (LF) and right flank (RF) correspond to flanking regions used in pUC57.VP30.mNeonGreen and highlight position on the ASFV parental virus genome. **ABii.** Design of pUC57 plasmid containing VP30mNeonGreen reporter cassette architecture, used for homologous recombination. **ABiii.** Regions of GeorgiaΔK145R to be targeted for deletion showing corresponding 500bp flanking regions. **ABiv.** The outcome of homologous recombination with deletion mutants showing viral genes replaced with Vp30mNeonGreen reporter cassette is shown. Note: Schematic is to scale. Schematic is adapted from (Rathakrishnan, Moffat et al. 2020). Generation of mutants was performed by Ana Reis and Anusyah Rathakrishnan (The Pirbright Institute).

8.2.2 GeorgiaΔMGFAK145R Showed an Impaired Ability to Inhibit the Expression of IFN Stimulated Genes

In vitro assessment of the impact of GeorgiaΔMGFAΔK145R and GeorgiaΔMGFBΔK145R deletion mutants upon expression of ISG was assessed relative to parental virus Georgia07/1, and well described attenuated isolate OURT88/3 which, relative to Georgia07/1, lacks MGF360-10L, 11L, 12L, 13L, 14L, MGF505-1R, 2R and 3R (Chapman, Tcherepanov *et al.* 2008, Chapman, Darby *et al.* 2011). Purified porcine bone marrow cells were infected with either mock, Georgia07/1, GeorgiaΔMGFAΔK145R, GeorgiaΔMGFBΔK145R, or OURT88/3 at an MOI of 2 and lysed at 0 hours post infection (hpi), 2 hpi, 8 hpi, 16 hpi or 24 hpi. Using qPCR analysis according to methods (See 2.9). Infections were performed in biological duplicate, and qPCRs in technical duplicates. I profiled changes in expression of 4 different ISGs, MX1, MX2, CXCL10 and PKR relative to housekeeping endogenous control GAPDH (Figure 8-2). As expected, no viral isolates induced a significant increase in levels of these mRNAs compared to mock-infected cells at 0 hpi. The most striking result revealed was that at 2 hpi, compared to mock, GeorgiaΔMGFAΔK145R induced a significant dramatic increase in relative expression of MX1 (14.6 (p=0.0023)), MX2 (9.8 (p≤0.0001)), CXCL10 (26.9 (p=0.0078)), and PKR (11.6 (p=0.0043)), whereas this was not the case for Georgia07/1, or GeorgiaΔMGFBΔK145R. OURT88/3 induced a significant rise in transcripts for MX1 (11.3 (p=0.0260)), and MX2 (5.35 (p=0.0059)), a substantial but not significant rise in CXCL10 (13.45 (n.s)), but little change in PKR expression (1.85 (n.s)). In contrast GeorgiaΔMGFBΔK145R and Georgia07/1 induced modest non-significant fold changes in all 4 ISGs,

(GeorgiaΔMGFBΔK145R: MX1; 5.5 (n.s), MX2; 2.6 (n.s), CXCL10; 1.7 (n.s), PKR; 2.3 (n.s). Georgia07/1: MX1; 4.3 (n.s), MX2; 2.7 (n.s), CXCL10 2.6 (n.s); PKR; 0.4 (n.s)). Likewise, I saw a significant increase in MX1 expression following infection with GeorgiaΔMGFAΔK145R and OURT88/3 at 8 hpi, but only OURT88/3 at 24 hpi (Figure 8-2A). MX2 expression was significantly higher with the same two isolates at 8 hpi and 24 hpi, although only OURT88/3 at 16 hpi (Figure 8-2B). In the case of CXCL10 expression, the fold change was much higher in OURT88/3 infected cells compared to GeorgiaΔMGFAΔK145R and other isolates from 8 hpi onwards, (8 hpi 29.2 vs. 9.1, 16 hpi 50.0 vs 8.1, 24 hpi 54.7 vs. 6.7). Furthermore, at 2 hpi OURT88/3 did not induce a significant increase compared to mock infections, whereas GeorgiaΔMGFAΔK145R did so (Figure 8-2C). Results for expression of PKR were more variable with GeorgiaΔMGFAΔK145R being by far the strongest inducer and the only virus to induce a significant increase over mock at 2 hpi. However, none of the viral isolates showed a significant increase of PKR over mock at 8 hpi, 16hpi, or 24 hpi (Figure 8-2D).

Conversely Georgia07/1 showed a non-significant induction of expression of all 4 genes at all time points. This was similar in manner to GeorgiaΔMGFBΔK145R. Furthermore, no significant difference between Georgia07/1 and GeorgiaΔMGFBΔK145R was observed for any ISG at any time point. Together this indicates a functional similarity between the wild-type virulent isolate and the non-attenuated deletion mutant.

Further work confirming these results is required. Investigation into the GeorgiaΔMGFAΔK145R vaccine strain's global impact on ISG expression would

be of interest, offering a potential understanding of how the deleted genes are controlling host transcription.

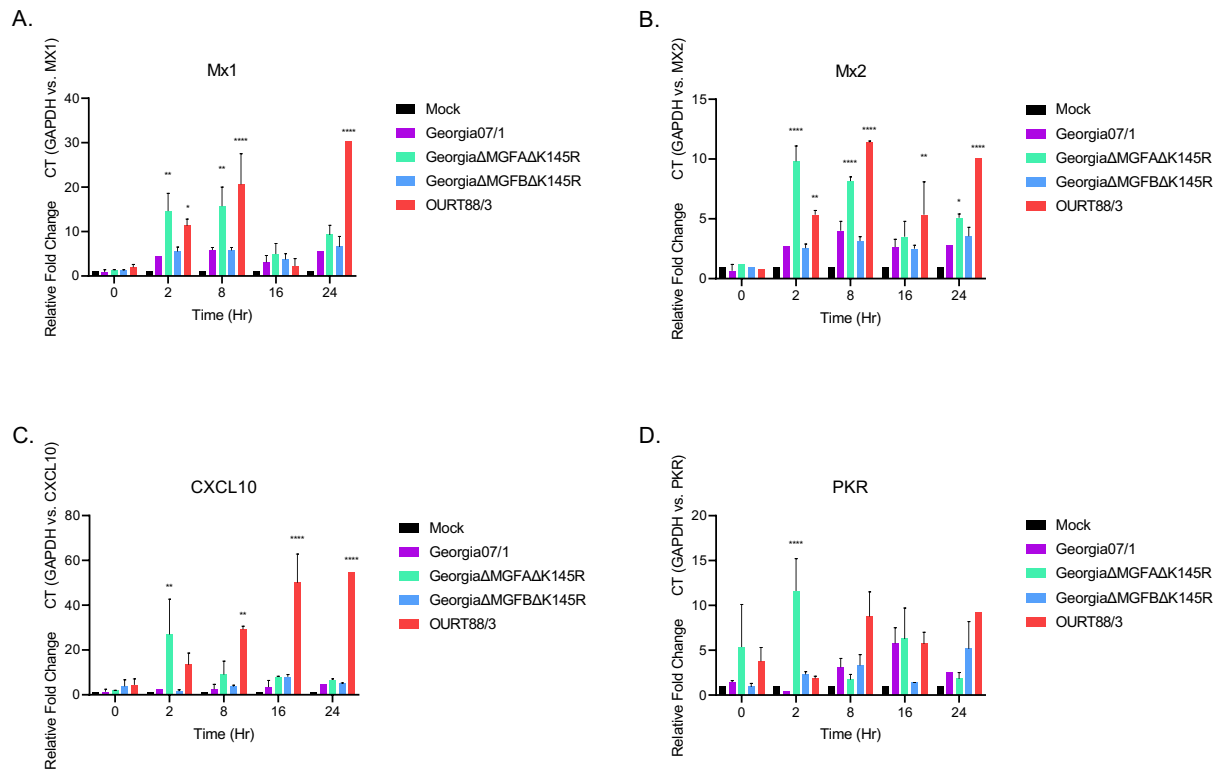


Figure 8-2. qPCR analysis of ISG expression at 0, 2, 8, 16, and 24 hpi. Porcine primary bone marrow cells were inoculated with either mock, wild-type Georgia07/1, naturally attenuated OURT88/3, or deletion mutants GeorgiaΔMGFAΔK145R, or GeorgiaΔMGFBΔK145R at a multiplicity of infection of 2. Cells were lysed at the indicated time points and RNA extracted. Expression of ISGs **A.MX1**, **B. MX2**, **C. CXCL10** and **D. PKR** was assessed by $\Delta\Delta C_t$ method relative to GAPDH endogenous control expression. Data is an average of biological duplicates, with each sample performed in technical replicate. Bars represent range of values. Tukey's test was applied for statistical analysis: n.s. = $p > 0.05$, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$.

8.2.3 In Vivo Challenge Study of Vaccine Candidates Showed that GeorgiaΔMGFAΔK145R Induced Protection Against Challenge with Georgia07/1, whereas GeorgiaΔMGFBΔK145R was not Attenuated

In vivo challenge experiments were performed at The Pirbright Institute's SAPO4 animal facilities. Inoculation of pigs, temperature assessment, clinical scoring and general husbandry of the animals was performed by the institute's animal service staff. Clinical scoring was assessed according to King, Chapman *et al.* (2011). Experiments were ethically reviewed and carried out under Government of the United Kingdom Home Office license.

12 large white landrace cross 8 week old female pigs over 20 kg were randomly divided into two groups of 6 (Group A - GeorgiaΔMGFAΔK145R, and Group B - GeorgiaΔMGFBΔK145R). After a period of acclimatisation, on day 0 Group A and B were intramuscularly (IM) immunised with 1×10^4 TCID₅₀ of respective vaccine candidates. Clinical scores including temperatures were recorded daily. Pigs in Group A were given a boost of 1×10^4 TCID₅₀ on day 21 and again on day 28 of 1×10^5 TCID₅₀. Animals were IM challenged with 1×10^3 TCID₅₀ Georgia07/1 on day 45 in parallel with a group of 3 non-immune pigs as control for the inoculum (Group C) which were brought in to facilities for acclimatisation 7 days prior to challenge. Animals were assessed for changes in temperature and clinical scoring daily as according to King, Chapman *et al.* (2011). Clinical scoring was based upon 10 different categories; each category was scored depending on

severity of symptoms. Categories were as follows (range of scoring shown in brackets): temperature (1-5), anorexia (1-6), lethargy (1-6), haemorrhagic rash (1-3), joint swelling (1-4), respiratory difficulties (1-4) ocular discharge (1), diarrhoea (1-4), bloody urine (4), and vomiting (4). Absence of symptom was scored as 0. Animals were euthanized on reaching a moderate-severity humane endpoint as described in the project license. Animals that did not require premature culling were euthanised at day 65. See Figure 8-3 for challenge study protocol.

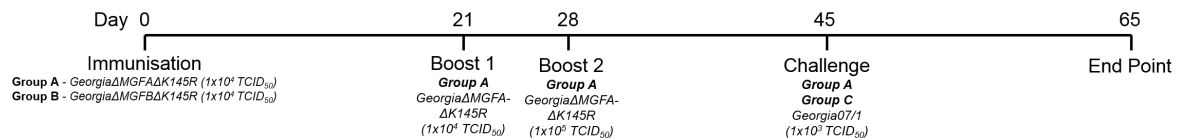


Figure 8-3. Overview of in vivo challenge study Immunisation was performed on day 0, and study continued until proposed endpoint on day 65. **Group A** were IM vaccinated with *GeorgiaΔMGFAΔK145R* (1×10^4 TCID₅₀) on day 0 and given a further intramuscular boost on day 21 (1×10^4 TCID₅₀) and day 28 (1×10^5 TCID₅₀). **Group B** were IM vaccinated with *GeorgiaΔMGFBΔK145R* (1×10^4 TCID₅₀) on day 0. Group A, and naïve control **Group C** were challenged with wild-type *Georgia07/1* (1×10^4 TCID₅₀) IM on day 45.

Group A animals showed 100% survival after immunisation (Figure 8-4). Whereas in Group B all 6 animals reached humane endpoint within 10 days of vaccination prior to challenge. Four animals of Group A survived until the end of the experiment (66.6% survival post-challenge) (Figure 8-4). After infection with GeorgiaΔMGFBΔK145R, all animals of Group B showed a rapid spike in temperature, from a physiologically typical baseline of 38.5-40°C, after the infection beginning at approximately day 5, with temperatures peaking between 41°C and 42°C (Figure 8-5B). Similarly, clinical scores reflected this with pigs showing a sudden increase around day 5, peaking 3-4 days later (Figure 8-6B). All animals had required premature euthanasia by day 10 post immunisation.

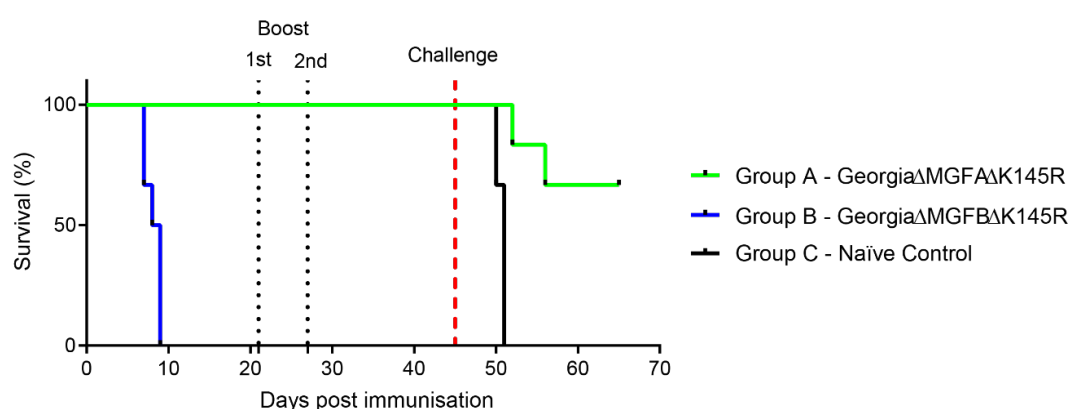


Figure 8-4. Survival rates of Group A, B and C The x-axis shows days post immunisation. The y-axis shows the percentage of animals that survived post immunisation (day 0) and/or challenge (day 45 - red dashed line) until endpoint on day 65. **Group A** (green line), 6 animals were IM immunised with 1×10^4 TCID₅₀ GeorgiaΔMGFAΔK145R on day 0 and boosted (black dashed lines) with the same deletion mutant on days 21 (1×10^4 TCID₅₀) and 28 (1×10^5 TCID₅₀). **Group B** (blue line), 6 animals were IM infected with 1×10^4 TCID₅₀ GeorgiaΔMGFBΔK145R on day 0. Group A and 3 naïve control animals (**Group C** – black line) were IM challenged with 1×10^3 TCID₅₀ Georgia07/1 on day 45.

Animals of group A did not show an observable trend of an increase in temperature post immunisation, or post either the first or second boost (Figure 8-5A). All pigs in Group A showed an increased temperature post-challenge. Animals A1 and A4 temperatures peaked at 41.7°C on day 55, and 42.0°C on day 51 respectively. Both animals A1 and A4 showed fever above 40.5°C for 3 consecutive days and were euthanised at the humane endpoint. All other animals returned to pre-challenge temperatures after a transient fever. Other clinical signs remained low until challenge (Figure 8-6A). After challenge 4 pigs showed transient mild lethargy, whereas A1 and A4, the same individuals with high temperature, showed high clinical scoring and required euthanasia. Animals A2, A3, A5 and A6 all reached the end of the experiment. As expected, Group C control animals showed a sudden spike in temperature, peaking at approximately 5 days post challenge (Figure 8-5C.). This was reflected in clinical scoring which had similar results (Figure 8-6C.). All animals of Group C reached the predetermined humane endpoint threshold and required euthanasia by 6 days post challenge.

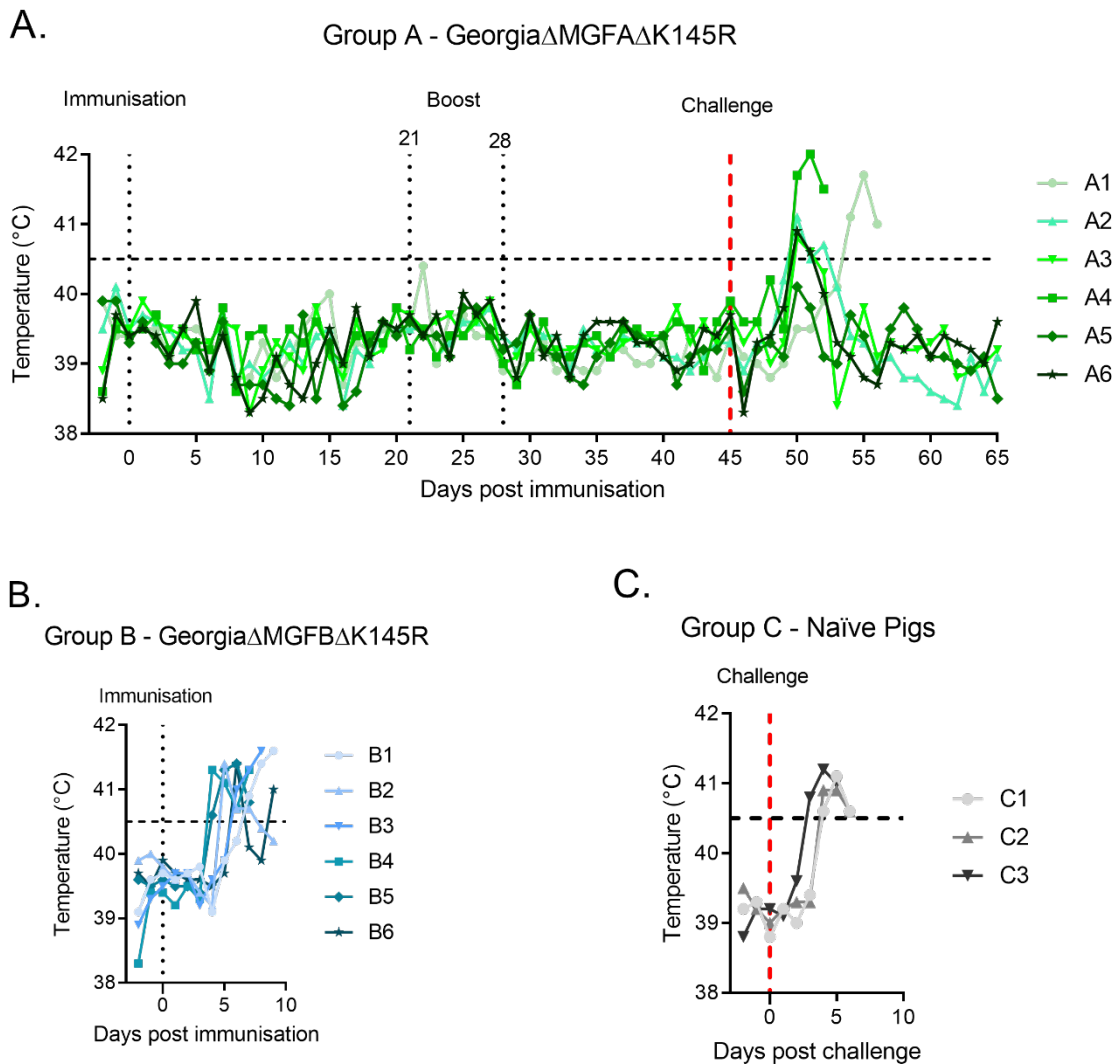


Figure 8-5. Temperature scoring of individual animals throughout the study

Temperatures were measured by Pirbright Institute animal staff. Humane endpoint threshold was met if temperature exceeded 40.5°C for 3 days, although clinical scoring was also taken into consideration. Dashed horizontal bar indicates temperature threshold. **A.** Shows temperature of individual animals from group A (vaccinated with Georgia Δ MGFA Δ K145R) across multiple days post immunisation. **B.** Shows temperature of individual animals from group B (vaccinated with Georgia Δ MGFB Δ K145R) across multiple days post immunisation. **C.** Shows temperature scoring of individual naïve control animals from group C post challenged with Georgia07/1.

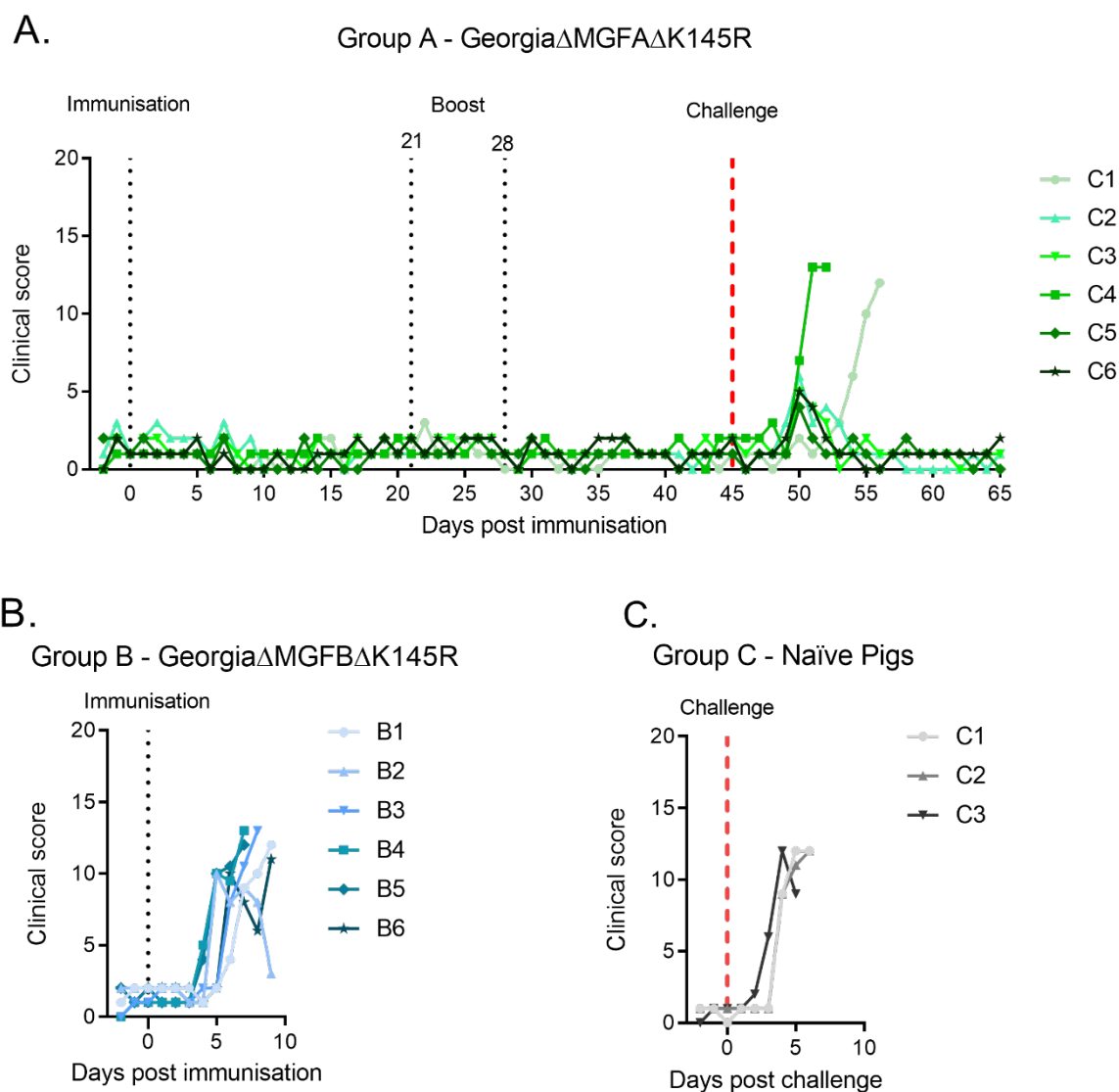


Figure 8-6. Clinical scoring for individual animals per day throughout the study

Clinical scores were assessed by Pirbright Institute animal staff as described by King, Chapman et al. (2011). **A.** Shows clinical score of individual animals from group A (immunised with Georgia Δ MGFA Δ K145R) across multiple days post immunisation. **B.** Shows clinical scores of individual animals from group B (immunised with Georgia Δ MGFB Δ K145R) across multiple days post immunisation. **C.** Shows clinical scoring of individual naïve control animals from group C post challenged with Georgia07/1.

8.2.4 Vaccination with GeorgiaΔMGFAΔK145R did not Provide Sterile Immunity

In order to assess viraemia, blood samples were collected by Pirbright Institute Animal staff from each animal once prior to immunisation, and regularly thereafter until termination on day 65. Samples were used for qPCR analysis of viraemia as determined by viral genome copies per mL of blood. qPCR analysis of viral genome copies per mL of blood was based upon mRNA copy numbers of the B646L viral gene (encoding VP72 protein) as described in (King, Reid *et al.* 2003), and performed by Anusyah Rathakrishnan. This analysis revealed that there was a transient low-level replication of GeorgiaΔMGFAΔK145R in a single animal above the limit of detection (LOD) ($10^{2.2}$ genome copies per mL), animal A5 at day 6 post immunisation, with approximately $10^{2.7}$ genome copies per mL (Figure 8-7A). Viral load of the vaccine strain was resolved in animal A5 by the next sampling on day 14 (Figure 8-7A). All Group B animals however showed a peak genome copies at a range of $1 \times 10^{6.9}$ - $1 \times 10^{7.8}$ genome copies per mL at euthanasia (Figure 8-7B). This was comparable to the number of genome copies observed post challenge with wild-type virus in Group C control pigs ($1 \times 10^{8.2}$ - $1 \times 10^{8.3}$ genome copies per mL) (Figure 8-7C), indicating that the gene deletions in GeorgiaΔMGFBΔK145R did not pose severe limitations on reproductive ability of the virus strain.

Relative to Group B, Group A GeorgiaΔMGFAΔK145R vaccinated animals showed significantly fewer genome copies post-immunisation. No increase in genome copies were observed post first or second boost with GeorgiaΔMGFAΔK145R

(1×10^4 TCID₅₀, and 1×10^5 TCID₅₀ respectively) in Group A (Figure 8-7A). Taken together this suggests that the deletion of MGF360-12L, 13L, 14L and MGF505-1R in GeorgiaΔMGFAΔK145R was sufficient to severely impair but not fully abrogate viral reproduction; whereas as replication was observed post immunisation with GeorgiaΔMGFBΔK145R, therefore deletion of MGF360-13L, 14L, MGF505-2R and 3R was not sufficient to abrogate replication. As expected, the 3 naïve control Group C animals, all showed a sudden rise of genome copies post challenge, with titres increasing to peaks of $1 \times 10^{8.3}$ genome copies per mL at 5 or 6 days post challenge coinciding with termination (Figure 8-7C). After challenge however an increase in viral load was clearly observed in all animals of Group A, with pigs A1 and A4 reporting the highest peak of $1 \times 10^{7.5}$ genome copies per mL at day 56, and $1 \times 10^{6.8}$ genome copies per mL at day 52 respectively (Figure 8-7A). This was not unexpected as these two animals displayed the most severe pathology of the group post challenge. For the remaining 4 pigs genome copies peaked at day 52 ($1 \times 10^{4.0}$ to $1 \times 10^{5.4}$ copies per mL) and continued to decline until experiment end point on day 65 (Figure 8-7A). This therefore revealed that the protection induced by GeorgiaΔMGFAΔK145R is not sterile immunity.

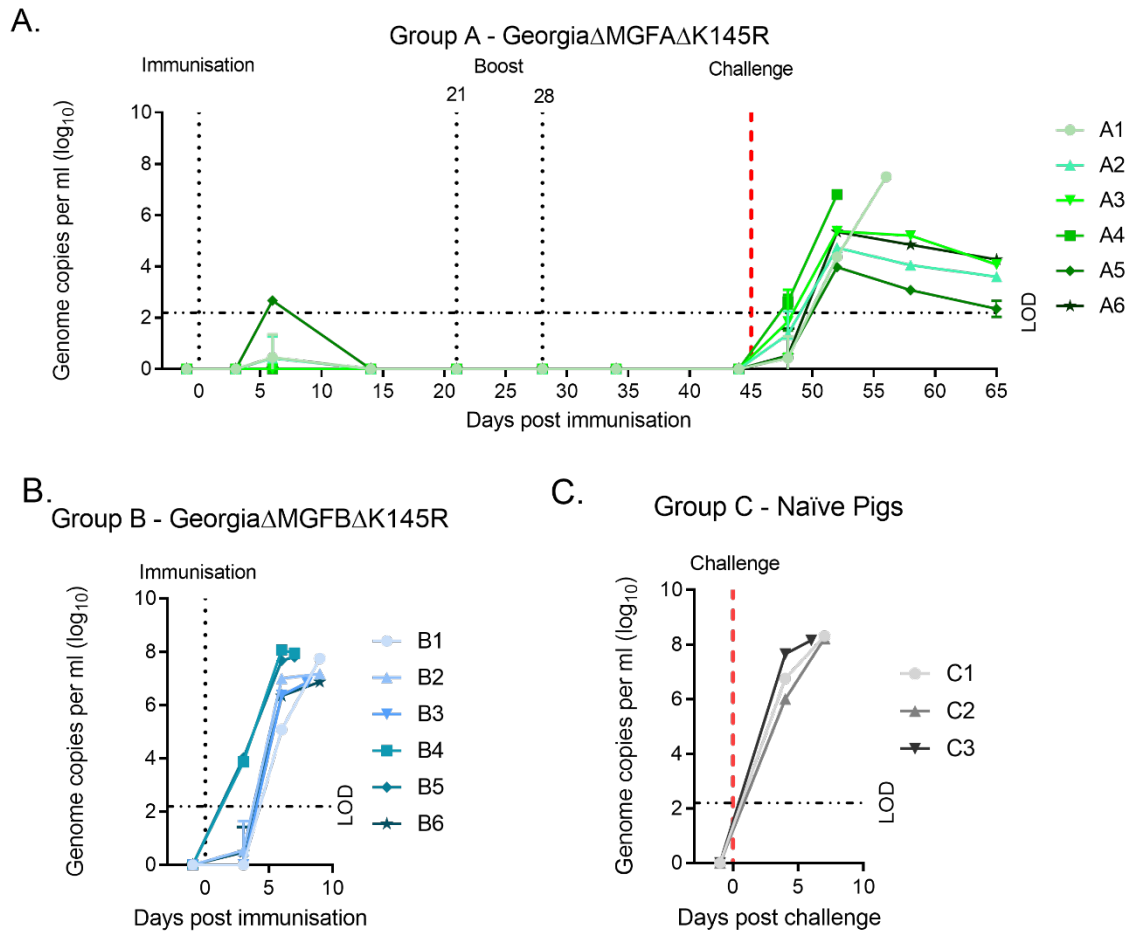


Figure 8-7. qPCR determined viral genome copies in blood samples Genome copies detected per mL ($\log_{10}$) of blood samples in comparison to a standard reference VP72 plasmid as described by (King, Reid et al. 2003). Analysis was performed by Anusyah Rathakrishnan. **A.** Total genome copies per mL ($\log_{10}$) Group A pigs vaccinated with GeorgiaΔMGFAΔK145R for the duration of the study **B.** Genome copies per mL of blood ($\log_{10}$) for Group B pigs vaccinated with GeorgiaΔMGFBΔK145R. **C.** Genome copies per mL ($\log_{10}$) for Group C control group post challenge with Georgia07/1.

8.3 Discussion

Numerous previous reports have provided insight that the MGF360-10L, 11L, 12L, 13L, 14L and MGF505-1R, 2R, and 3R play an important role in ASFV virulence, that their collective deletion attenuates the virus and these deletion mutants show promise as potential vaccine candidates (See 1.2.6) (Boinas, Hutchings *et al.* 2004, King, Chapman *et al.* 2011, O'Donnell, Holinka *et al.* 2015, Reis, Abrams *et al.* 2016, Chen, Zhao *et al.* 2020). The vast majority of work investigating the impact of these genes on viral attenuation has focused upon the deletion of the entire group or a large portion of, whereas little is known of the impact of individual genes on attenuation. I aimed to illuminate which individual genes were responsible for attenuation. Therefore, the rationale of having overlapping gene deletion profiles of Georgia Δ MGFA Δ K145R and Georgia Δ MGFB Δ K145R was to allow us to pinpoint towards the individual genes responsible for attenuation.

In this chapter the impact of overlapping deletions of 4 MGF genes on transcription of ISGs was investigated. Two gene deletion mutants were constructed by The Pirbright Institute's ASFV Group colleagues, Ana Reis and Anusyah Rathakrishnan; Georgia Δ MGFA Δ K145R lacking MGF360-12L, 13L, 14L, MGF505-1R and K145R; and Georgia Δ MGFB Δ K145R lacking MGF360-13L, 14L, MGF505-1R, 2R and K145R. I investigated the impact of the deletions on transcription of ISGs by infecting primary porcine bone marrow cells with the Georgia Δ MGFA Δ K145R, Georgia Δ MGFB Δ K145R, Georgia07/1 or OURT88/3, and measuring transcript levels of MX1, MX2, CXCL10, and PRK mRNA expression. The results showed that Georgia Δ MGFA Δ K145R was capable of inducing a rapid

increase of MX1, MX2, CXCL10 and PKR expression early in infection in a pattern that was more reminiscent of attenuated OURT88/3 than Georgia07/1 or non-attenuated deletion mutant Georgia Δ MGFB Δ K145R (Figure 8-2). The difference however was less obvious at later time points. As the replication cycle of ASFV is complete at approximately 18-24 hpi (Muñoz-Moreno, Galindo *et al.* 2015), cells at the 24 hpi may also be undergoing a second round of infection by progeny virus at the early stage. Furthermore, the temporal expression profile of inhibitory genes may impact on the time required to subvert the ISG response. ASFV shows a temporal expression profile (Rodríguez, Salas *et al.* 1996, Rodríguez and Salas 2013, Cackett, Matelska *et al.* 2020), and many MGFs are thought to be early expressed (Cackett, Matelska *et al.* 2019), the reduced ISG expression with Georgia Δ MGFA Δ K145R at later timepoints therefore may be explained by inhibitory action of late expressed proteins coming into effect. VP72 qPCR assay assessing viral titres at different time points would be of significant interest as variation in reproductive capability may have an impact upon the results at different times.

Interestingly genes of the MGF360 and MGF505 family have been implicated in altering the expression of these ISGs. Afonso, Piccone *et al.* (2004) reported that cells infected with the ASFV isolate Pr4 Δ 35 lacking MGF360-9L, 10L, 11L, 12L, 13L, 14L and MGF505-1R and 2R showed an upregulated expression of MX1, MX2, PKR and a number of other ISGs relative to wild-type isolate Pr4. Fishbourne, Hutet *et al.* (2013) reported that *in vivo* CXCL10 mRNA expression has been found to be elevated in blood from pigs infected with virulent Benin97/1

isolate, at a level higher than that of low virulent OURT88/3 at least 3 days post infection. The authors proposed that as ASFV replicates in macrophages, higher CXCL10 may promote chemotaxis of macrophages towards the site of infection to facilitate further viral replication. This was not the case with the *in vitro* study here, in fact I observed the opposite that in attenuated isolates OURT88/3 and GeorgiaΔMGFAΔK145R mRNA expression level was considerably higher than virulent isolate Georgia07/1. This difference may be explained by the complex response seen in *in vivo* conditions which includes the impact of bystander cells, therefore making a direct comparison is exceptionally difficult. Furthermore, in contrast to *in vitro* data, virulent isolates have been shown to be capable of inducing high levels of IFN in sera from infected animals (Golding, Goatley *et al.* 2016), this may explain the results seen by Fishbourne, Hutet *et al.* (2013). Quantification of the actual levels of ISG proteins produced on viral infection by enzyme-linked immunosorbent assay (ELISA) is required. Although the results here indicate that GeorgiaΔMGFAΔK145R is a strong early inducer of ISG expression, it has to be taken into careful consideration that inoculum was cell media containing the virus and therefore may have contained residual cytokines e.g. IFN. Considering that the induction of ISG expression requires JAK/STAT signalling pathway induction the observed response was earlier than expected. This may be explained by expression driven by IRF3 or NFκB transcription factor activation in response. For instance, human cytomegalovirus infection, Mx1, Mx2 and CXCL10 have been shown to be upregulated independently of IFN and JAK/STAT, but in an IRF3-dependant manner (Ashley, Abendroth *et al.* 2019). Additionally, according to transcription factor analysis tool, Interferome database v2.01, Mx2 promoter contains an IRF3 binding region, PKR promoter an NFκB

binding region, whereas CXCL10 promoter contains both (Rusinova, Forster *et al.* 2012). Rapid induction of these genes therefore may be explained by IRF3 or NFκB driven induction. To eliminate the effect of residual IFN, viral stocks should be tested for the cytokine by ELISA, or a broader approach with multiplex cytokine assays to assess multiple inflammatory markers simultaneously. Additionally, work should be repeated after filtering viral stocks for debris and proteins.

Here the *in vivo* challenge results showed that the deletion of MGF360-12L, 13L, 14L and MGF505-1R along with K145R (GeorgiaΔMGFAΔK145R) attenuated genotype II Georgia07/1 and induced protection of 66.6% on challenge with the parental virus. GeorgiaΔMGFBΔK145R absent of MGF360-13L, 14L, and MGF505-2R and MGF505-3R however was not attenuated and displayed a phenotype typical of virulent ASFV isolates. This was concurrent with the qPCR findings suggesting that GeorgiaΔMGFAΔK145R induced ISG early expression, whereas GeorgiaΔMGFBΔK145R did not.

Vaccination with GeorgiaΔMGFAΔK145R did not induce pyrexia or a significant rise in clinical scoring post immunisation, or either boosts. It was not until challenge with Georgia07/1 that substantial pyrexia or appearance of clinical symptoms in the animals were observed, however these were resolved in all but 2 animals which met the humane endpoint threshold. Post challenge viraemia of challenge virus was 2-3 orders of magnitude lower than that observed in naïve pigs, indicating that protection was not sterile. Genome copies however continued to decline until experiment endpoint on day 65, therefore whether

animals would have completely cleared challenge virus given sufficient time remains to be seen. O'Donnell, Holinka *et al.* (2015) noted that immunisation with Georgia07/1 deletion mutant lacking MGF360-12L, 13L, 14L, and MGF505-1R, 2R, and 3R attenuated the virus and induced 100% protection with no signs of disease before challenge, however after challenge, up to 40% of animals continued to harbour the challenge virus which corresponded with fever. Similarly, Chen, Zhao *et al.* (2020) deleted the same profile of 6 MGFs (MGF360-12L, 13L, 14L, MGF505-1R, 2R, and 3R) from genotype II HLJ/18 backbone (highly similar to Georgia07/1). Immunisation with this deletion mutant did not cause pyrexia and provided 100% protection against HLJ/18, although 1 in 4 animals developed prolonged fever of up to 9 days. However it must be taken into account that variation in strain backgrounds, and study design, (such as different boost and challenge schedules, or different immunisation and challenge titres), mean that it is extreme difficulty to make a direct comparison between *in vivo* challenge studies.

In contrast to immunisation with Georgia Δ MGFA Δ K145R, animals immunised with Georgia Δ MGFB Δ K145R showed typical pathology for domestic pigs infected with virulent ASFV. Analysis of viraemia of this vaccine strain performed by Anusyah Rathakrishnan also showed high titres consistent with that of wild-type virus of the same background. Taken together this revealed that the deletion of MGF360-13L, 14L and MGF505-2R and 3R was insufficient in attenuating viral virulence and did not reduce rates of viral replication *in vivo*. As both deletion candidates tested contained an overlap in profiles lacking MGF360-13L and 14L,

taken together with the *in vivo* data demonstrating attenuation of GeorgiaΔMGFAΔK145R but not GeorgiaΔMGFBΔK145R we can deduce that the individual gene, or pair of genes, responsible for attenuation is one, or both, of the two additional genes absent from GeorgiaΔMGFAΔK145R: MGF360-12L and MGF505-1R.

A major consideration of LAVs is their safety. It is of concern that Chen, Zhao *et al.* (2020) reported that during repeated passages of a deletion mutant virus lacking 6 MGFs through pigs, the virus progressively replicated more efficiently with an apparent reversion to lethality by passage 5. Due to the MGFs being thought to arise from gene duplication, potentially in a gene accordion type mechanism, I posit that that gene expansion of remaining MGFs could have occurred to compensate the loss of virulence caused by the gene deletion. However, the newly virulent vaccine derived virus was disappointingly not sequenced, therefore it cannot be conclusively said that the phenotypic change was due to this mechanism. Importantly, this clearly establishes a strong imperative to perform work to eliminate this as a cause for the reversion to virulence.

The data shown here suggests that MGF360-12L and MGF505-1R are responsible for inducing ISG expression and attenuation in swine matches my previous work indicating these genes act as potent inhibitors of the IFN signalling pathway (See Chapter 5 and 6), thus their absence would allow the host to mount a sufficient immune response. However, as the profile of genes absent in

Georgia Δ MGFB Δ K145R were also shown to be inhibitors of the IFN pathway, it is curious as to why inhibition of ISG expression and attenuation appears to be dependent upon MGF360-12L and/or MGF505-1R. The answer is not clear, but this may simply be explained by one of these proteins playing a much more significant role in immune subversion than the others. As I have shown that compared to the other MGFs of interest MGF360-12L plays a unique multifunctional role to inhibit the IFN pathways at numerous levels (See Chapter 5, 6 and 7). Therefore, it may be that the combined effect of a multipronged attack on the IFN signalling pathway in the presence of MGF360-12L compensates for the loss of MGF360-13L, 14L, MGF505-2R and 3R in Georgia Δ MGFB Δ K145R, thus explaining its continued virulence. Additionally, it may also be possible that MGF360-12L, and MGF505-1R are acting upon the immune response in a cumulative or synergistic manner, or are doing so with MGF360-13L and 14L, thus their loss may result in the attenuation of Georgia Δ MGFA Δ K145R. Generation of gene deletion mutants solely lacking MGF360-12L or MGF505-1R, or both, would be of obvious interest. *In vitro* IFN stimulation assays and further *in vivo* challenge studies using these single gene deletion viruses would provide deeper insight into which of the two genes is responsible, or whether multiple gene deletions are required for attenuation.

9 DISCUSSION

ASF poses an extreme threat to regions that rely on pork production. No vaccines are currently available, leaving only prevention or extermination measures as methods of control. As the virus continues to ravage across Europe and East Asia, a vaccine is therefore crucial in the fight against the disease. Here I have built upon the body of work showing that genes from the MGF360 and MGF505 cluster are inhibitors of the IFN pathway, important host range determinants, and clearly responsible for the attenuation of virulent isolates (see 1.2.4). However only very limited work has been performed to assess the functional characteristics of individual members for the two clusters (Zhuo, Guo *et al.* 2020). Here I have revealed new data that gives insight into the individual functionality of MGF360-10L, 11L, 12L, 13L, 14L, MGF505-1R, 2R, and 3R genes.

The investigation began with computational analysis including sequence motif analysis and putative structural homology modelling. This revealed the potential presence of ANK repeat motifs in the majority of the proteins of interest, but also the presence of a previously unseen novel SOCS/VHL-Box-like motif (See 3.2.3). ANK repeats are highly abundant throughout eukaryotic proteins but rare elsewhere, with the exception of poxviruses which are thought to have acquired the motif by horizontal gene transfer (Herbert, Squire *et al.* 2015). As ASFV and poxviruses both belong to the NCLDV family it is therefore unsurprising to uncover these motifs. Interestingly this may point to the acquisition of genes coding for ANK proteins by a common ancestor, although an independent gene

acquisition event may also explain their presence in ASFV. The SOCS/VHL-Box motifs are found in substrate binding proteins are responsible for the recruitment of CRL machinery in a modular like fashion (See 1.1.7.4) (Kamura, Sato *et al.* 1998, Kamura, Maenaka *et al.* 2004). Substrate binding proteins act in a bipartite manner, the CRL functions as a E3 Ub ligase targeting substrate bound to binding motifs for ubiquitination and proteasomal degradation. Interestingly poxvirus' PRANC proteins use ANK repeats to mediate substrate binding, and contain an F-Box, a motif highly similar to SOCS/VHL-Box to drive recruitment of the CRL (See 1.1.7.4) (Sonnberg, Seet *et al.* 2008). It is however unusual for SOCS/VHL-Boxes to be associated with ANK repeats in poxvirus proteins, which are typically found in conjunction with F-Boxes. There are exceptions however, for example a novel class of orthopoxvirus proteins contain the BC-Box portion of the VHL/SOCS-Box, but unlike the MGFs not reported to contain the full motif (Odon, Georgana *et al.* 2018). Likewise cellular ANK-SOCS-Box (ASB) proteins contain both as the name suggests (Anasa, Ravanan *et al.* 2018). The impact of the MGFs on the IFN response, coupled with the discovery of motifs and protein architecture that is reminiscent of poxvirus PRANC proteins, provided the investigative platform to understand the functional mechanism of the viral proteins. Consequently, I hypothesised that the MGFs may be using their N-terminal SOCS/VHL-Box-like motifs to recruit CRL machinery in order to target substrate bound to ANK repeats. Due to the known function of inhibiting the IFN response, it would not go beyond reasonable doubt to suggest that the substrate may be a component of the IFN signalling pathways.

On that basis, yeast-2-hybrid assays were performed probing the MGFs of interest for interactions with components of IRF3 and NFκB signalling pathways.

This revealed a number of interactions including MGF360-12L with TRAF3, and TRAF6 a key component in NF κ B signalling, moreover this hinted that the putative novel TRAF6 binding motif found within the protein during the bioinformatic analysis may be functional. Curiously however, further examination by CoIP did not pulldown the cellular protein with MGF360-12L. A potential explanation for this is that if the interaction is unstable or short-lived. Therefore, further assessment is needed to confirm the yeast-2-hybrid results. Although mutagenesis of the putative novel TRAF6 binding motif did partially alleviate inhibition of the luciferase reporter under the control of the NF κ B response element indicating that the motif does play a role in its inhibitory mechanism.

In order to assess potential inhibition of the type-I IFN signalling pathways by the MGFs, I performed luciferase assays to probe along the pathways. IRF3 and NF κ B pathways were probed at key stages, including PRR and cytokine receptors stimulation (via Sendai virus, or TNF α /IL-1 β respectively), mid-level kinases (exogenous expression of TBK1, or IKK α /IKK β) and also at the level of the transcription factors (exogenous IRF3-5D or p65 expression). This revealed that MGFs were capable of inhibiting both the IRF3 and NF κ B pathways at the transcription factor level or below. Interestingly this was similar to the orthopoxvirus proteins with a similar VHL/SOCS-Box architecture (Odon, Georgana *et al.* 2018). Furthermore, MGF360-12L and MGF505-1R inhibition of NF κ B was in a dose dependent manner, whether this is also true for IRF3 inhibition remains to be seen and could be answered with additional luciferase assays. However, interestingly using a Gal4 luciferase system with the

transcription factors fused to gal4 DBD only MGF360-12L inhibited luciferase expression, hinting towards a functional impact directly upon the activity of these two transcription factors.

Subsequent work was focused on the specific molecular mechanisms of inhibition of MGF360-12L, and MGF505-1R. CoIP and western blotting revealed that neither viral protein interacted with or impacted upon the activation of NFκB; confocal microscopy confirmed this as I observed p65 nuclear translocation on stimulation with TNFα. In contrast to NFκB, MGF360-12L partially limited IRF3 nuclear translocation on stimulation by Sendai virus, but did not interact with or clearly impact the total protein level of the transcription factor. I was however unsuccessful in attempts to probe for IRF3 phosphorylation, a critical stage in the activation of the transcription factor. The question whether the viral protein may be interfering with IRF3 phosphoactivation remains, and work is clearly required to assess this in the first instance. Furthermore, for western blots and CoIPs, transfection efficacy should be confirmed, as insufficient viral protein may conceal an effect on endogenous proteins. Very recent work by Zhuo, Guo *et al.* (2020) reported MGF360-12L inhibited both IRF3 and NFκB pathways as shown here, but contrary to my findings blocked p65 nuclear translocation in HeLa cells treated with TNFα. Here I showed that MGF505-1R did not appear to affect either IRF3 levels or nuclear translocation, this coupled with the inhibition of constitutively active IRF3-5D luciferase assays indicates that the functionality of the IRF3 pathway remained intact, and that inhibition is occurring beneath the activation level of IRF3. Whether the viral proteins are preventing the transcription

factors binding to promoters' DNA remains to be seen and could be addressed by performing EMSA assays.

CoIP with mutant variants of the SOCS/VHL-Boxes revealed that the motifs were indeed functional and responsible for binding to CRL machinery. After initially finding the motifs, the ambiguity of the sequences in the MGFs meant it was difficult to determine whether they were VHL-Box or SOCS-Box motifs, or a hybrid of the two. Both motifs are closely related but recruit CRL machinery via different Cul scaffold proteins, Cul2 and Cul5 respectively (Kamura, Maenaka *et al.* 2004). MGF360-12L co-immunoprecipitated with Cul2, EloB and EloC, but not Cul5, indicating that the motif is functionally a VHL-Box. Whereas MGF505-1R pulled down endogenous EloB, and exogenous Cul5 but importantly not exogenous Cul2, demonstrating that the motif was likely functioning as a SOCS-Box. Mutation of key residues across the full length of the motifs inhibited MGF360-12L and MGF505-1R pulling down the CRL machinery, thus providing confidence that the motifs are crucial for the binding of Elongins and Cullins. Furthermore, in an attempt to understand as to whether this recruitment of the CRL is vital to the viral proteins inhibitory function, luciferase assays were performed with mutant variants. This revealed that mutation of the full length VHL-Box or its constituent parts (BC-Box and Cul-Box) of MGF360-12L did not alter functionality in inhibiting the NFκB and IRF3 pathways. Mutation of the SOCS-Box motif of MGF505-1R however did alleviate inhibition of the IRF3 and NFκB pathways. Mutation of the individual components of the motif revealed that MGF505-1R's inhibition of the IRF3 pathways is reliant on the full SOCS-Box motif, as inhibition remained with

mutation in BC-Box or Cul5-Box alone. In contrast, NFκB inhibition was alleviated with BC-Box and Cul5-Box mutations, albeit not to the same extent as the full motif. Taken together this indicated that the SOCS-Box is essential for MGF505-1R functionality, although intriguingly the VHL-Box despite being functionally active, is dispensable for MGF360-12L activity. This suggests that the motif may play an additional as yet unknown role, adding further weight to the idea that MGF360-12L acts with distinct multifunctional roles. Taken together it would suggest that the mechanism of action of these two viral proteins are distinct.

I hypothesised that MGF505-1R is co-opting the CRL machinery to target components of the IFN pathway. As inhibition was occurring at the transcription factor level or below for both the IRF3 and NFκB pathways, a component common to both was an obvious point of investigation. This included CBP/p300 cofactors essential for the formation of the enhanceosome and transcription of type-I IFNs (Kim, Kim *et al.* 1998, Merika, Williams *et al.* 1998, Honda, Takaoka *et al.* 2006). For instance, targeting of p300/CBP has been proposed as a mechanism of inhibition for a number of viruses, either preventing transcription factor interaction or displacing the cofactor from the promoter (Granja, Nogal *et al.* 2006, Granja, Sánchez *et al.* 2009, Ferrari, Gou *et al.* 2014, Zhang, Shi *et al.* 2016, Zemke and Berk 2017). Here I revealed by western blotting that MGF505-1R significantly reduced the level of endogenous p300, which did not occur with the SOCS-Box mutant variant. Therefore, it would appear that MGF505-1R may be using the CRL machinery to target the protein for degradation. If exogenous expression of p300 were to rescue inhibition of the pathways in the presence of

MGF505-1R this would add further evidence of its role in the mechanistic effects of the viral protein. Moreover, I was unable to show an interaction between the proteins using CoIP. *In situ* hybridised proximity ligation assay between MGF505-1R and p300 would offer a more sensitive approach to investigate any potential protein-protein interaction.

Additional evidence indicating an impact upon transcriptional activity was noted with MGF360-12L. The viral protein appeared to have an impact on the protein expression driven by some, but not all, exogenous plasmids, and did not appear to impact endogenous expression of the same proteins. Targeting of TFIIB component of the RNAPII complex by Thogoto virus is thought to limit *de novo* recruitment of RNAPII to preferentially inhibit antiviral gene expression but not broad spectrum transcription (Haas, Meiler *et al.* 2018). Despite not seeing an interaction between MGF360-12L and TFIIB, further work investing potential limitation of *de novo* IFN expression by the ASFV protein is warranted. Determination of which specific genes are inhibited by MGF360-12L by techniques such as transcriptome RNAseq analysis, may begin to shine light on a common mechanism or features of these genes that are being targeted, such as specific promoters. EMSA assay may also address whether the protein is binding to promoter specific sequences. An additional possible explanation for the activity of MGF360-12L may be linked to the VHL-motif itself. The EloA EloB EloC heterotrimer (Elongin (SIII)) interacts with RNAPII to strongly induce transcription by relieving transient pausing of transcriptional machinery, (Aso, Lane *et al.* 1995, Aso, Haque *et al.* 1996, Yasukawa, Sugimura *et al.* 2007),

although in times of cellular stress the complex induces the degradation of the Rpb1 (Yasukawa, Kamura *et al.* 2008). Interestingly this complex competes with the Von Hippel-Lindau protein from which the VHL-Box derives its name for EloB and EloC to control expression (Duan, Pause *et al.* 1995). Interestingly La Crosse NSs protein antagonises the IFN response by inducing Rpb1 degradation in an EloC dependant manner (Schoen, Lau *et al.* 2020). Although I did not see any degradation of Rpb1 or alteration in its phosphorylated status, whether or not MGF360-12L is competing with Elongin (SIII) would be an interesting avenue of investigation.

As I was unable to see interactions between MGF360-12L and MGF505-1R with potential substrate proteins, to address this a high throughput screening of proteomic interacting partners would be extremely interesting. This could be achieved by SILAC mass spectrometry and would hopefully reveal more details of the viral proteins' interacting partners and thus their mechanism of action. However, if the MGFs are targeting cellular proteins for degradation this may lead to insufficient quantities available for mass spectrometry, analysis with the mutant variants may be the better route of investigation to bypass this concern. In addition to this, as I saw inhibition of the NFκB and IRF3 pathways but also an increase in ISG expression with attenuated viral deletion mutant GeorgiaΔMGFAΔK145R, a similar investigation to the one performed here into whether the inhibitory impact of the MGFs extends further to additional pathways such as JAK/STAT pathway (See 1.1.6) would be of great interest.

Using the knowledge gained of the inhibitory function of the MGF360 and MGF505 clusters, I proposed two profiles of genes for deletion from virulent isolate Georgia07/1 with a previously performed deletion of K145R (Georgia Δ K145R) removed as a DIVA target, in order to generate a LAV candidate. This included deletion of MGF360-12L, 13L, 14L and MGF505-1R (Georgia Δ MGFA Δ K145R), and a second profile of MGF360-13L, 14L, MGF505-2R and 3R (Georgia Δ MGFB Δ K145R). Deletion of the genes was performed by homologous recombination and single cell sorting by Ana Reis and Anusyah Rathakrishnan (Rathakrishnan, Moffat *et al.* 2020). *In vitro* qPCR analysis in porcine macrophages revealed that Georgia Δ MGFA Δ K145R induced higher expression levels of ISGs MX1, MX2, CXCL10 and PKR than virulent Georgia07/1 at early time points. Georgia Δ MGFB Δ K145R however showed an expression profile more similar to that of the lethal wild-type virus. This potentially indicates that Georgia Δ MGFA Δ K145R has a reduced ability to inhibit the IFN response. ELISA assay quantifying protein levels is required to reveal whether the effect on expression observed here translates to impact on actual ISG levels.

Attenuation of Georgia Δ MGFA Δ K145R was confirmed in *in vivo* challenge studies. Vaccination with the isolate did not induce clinical pathology and protected 66.6% animals from challenge with parental virus. Georgia Δ MGFB Δ K145R was not attenuated however, inoculation with the deletion mutant showed a pathology typical of that of virulent ASFV and all animals were euthanised within 10 days of infection. This was lower than the 100% protection previously reported with more expansive deletions of MGFs

from Benin97/1 (Reis, Abrams *et al.* 2016), and Georgia07/1 (O'Donnell, Holinka *et al.* 2015) and HLJ/18 (Chen, Zhao *et al.* 2020). Relative to these gene deletion mutants, the loss of fewer MGF genes in GeorgiaΔMGFAΔK145R would be expected to be “less attenuated” and therefore more likely to induce a robust immune response, as to why this conferred less protection remains unclear. Although genotype background differences and variation in study design may partially explain the differing outcomes. Deletion of genes from seemingly responsible for immune modulation as in GeorgiaΔMGFAΔK145R may consequently increase the host IFN response, but additionally the virus' susceptibility to the cytokine thus impeding its replicative ability. Therefore, performing viral growth curves would be of interest to assess whether the deletions have any impact on replication. Furthermore, analysis of immune responses to the vaccine relative to virulent wild-type would be of interest and may provide an understanding of how the gene deletion isolate is inducing protection. Measurement of the T cell response could be achieved via IFN γ enzyme-linked immune absorbent spot (ELISpot) assay, or quantification of specific immune cell populations using Fluorescence-activated cell sorting (FACS) assay. Whereas ELISA assay could be used to determine ASFV specific sera antibody response, or probe for specific cytokines such as INF α/β , IFN γ , IL-1 β , TNF α . Together, deeper insight into the specific profile of *in vivo* immune response elicited by GeorgiaΔMGFAΔK145R may offer further understanding of the protection provided. However, the scope of what this may reveal could be limited as very little is known about the specific correlates of protection with ASFV (Carlson, O'Donnell *et al.* 2016). Nevertheless Oura, Denyer *et al.* (2005) demonstrated that the depletion of CD8 α^+ lymphocytes post immunisation with

OURT88/3 alleviated protection on challenge, moreover transfer of passive antibodies has also been observed to provide protection (Onisk, Borca *et al.* 1994). Although limited, this evidence when taken together would suggest that both a cellular and humoral response may be required for protection. Understanding the specific profile of immune response required for protection would be of use as analysis could determine whether any response mounted to vaccination is appropriate or not, and thus help future vaccine candidate design.

As the two vaccine candidates had an overlapping deletion profile (both were absent for MGF360-13L and 14L), I could infer from these results that attenuation of Georgia Δ MGFA Δ K145R was dependant on MGF360-12L and/or MGF505-1R. Future work creating deletion mutants solely lacking these individual genes would allow us to further investigate whether an individual gene or synergy between the two is required for the attenuation seen here. Simple qPCR analysis of the induction of IFN α / β would give an initial insight, likewise *in vivo* experiments would reveal whether attenuated or not. FACS to assess individual cells populations would limit the impact of “bystander effect” on expression changes. RNAseq analysis of the *in vivo* pig expression profile to the different gene deletion mutants would be of great benefit to begin to understand how the attenuated viruses impact upon expression of antiviral genes.

The data presented here provides the first insight into the molecular mechanisms of the MGF360 and MGF505 proteins in IFN inhibition, with a specific focus on MGF360-12L and MGF505-1R. Overall it would appear that both proteins act at

the level of the transcription factor or below with interference of transcriptional activity seemingly the general area of focus. Moreover, the deletion of these two MGFs is crucial in the attenuation of the Georgia07/1 isolate, demonstrating their importance to viral virulence. Furthermore, the data suggests that MGF360-12L deploys multiple functions or mechanisms of inhibition, potentially explaining the importance to viral subversion of the IFN system. This body of work provides a good platform to build upon to further define the precise mechanisms of action of these genes, but also adds valuable data to the development of much-needed ASFV vaccines.

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11 SUPPLEMENTARY FIGURES

Protein	Residues	Protein Hit [Species] (Ascension No.)	Identity (%)	Alignment
MGF360-10L	60-97	Ankyrin partial [Anaeromyces robustus] (ORX75274.1)	45	Query 60 TALMLATKENNYQLIKLFTEWGADINYGLICANTPPVR 97 TAL+ AT+ NN++++KL E+GA+IN G+ + PP++ Sbjct 44 TALIYATENNNFEIVKLLVEYGANINVGMY-HDGPPLK 80
MGF360-12L	40-86	LOW QUALITY PROTEIN: ankyrin repeat and SAM domain-containing protein 6 [Rhinatrema bivittatum] (XP_029445778.1)	44	Query 40 FDH-NLRLIKSAGIKEGLNLTALVKAVRENNYNLIKLF AEWGADINY 86 +DH ++ ++ S G +EGL TAL+ AV+ + ++ L EWGADINY Sbjct 182 YDHFDVNMVDSNGSREGLPDITALMTAVQHGHEAVVCLLLEWGADINY 229
MGF360-13L	99-159	tRNA preQ1(34) S- adenosylmethionine ribosyltransferase- isomerase QueA [Campylobacter lari] (EAJ5698718.1)	43	Query 99 HIRELCRQLGAKETLEDNDIFRIFTRIMHNKTS GSIILCHEIFMNNPILENKFVIQLRGLI 159 H ++L R L E + ND I RI NK SGS I E+F+N+P+ NKF++Q+RG * Sbjct 47 HFKDLARILSPCEIIF-NDTKVIKARIYGNKESGSKI---ELFINHPLKNNKFLVQIRGKV 103
MGF505-1R	142-193	Hypothetical protein [Helicobacter pylori] (WP_000346199.1)	33	Query 142 KYSKFRVLLYFKHRLEDDL PFTLLIEKACKDHNYEVIKWIYENLHIYNMID 193 K FR + L+DDL P L+E K++ Y+++KW+Y + + N +D Sbjct 4 KNKSFRSHAAAYTSTLKDDLPLNPNLVEAKTKENFYKLLKWLYSKMSVGNQVD 55
MGF505-2R	213-298	AAA family ATPase [Ferruginibacter sp. BO-59] (WP_123121017.1)	30	Query 213 LFDRGNTAATLLTQHLLKTAAGLLHFVLET LKYGGNIDTVLTQAVKYNHRKLLDYFLRQ 272 +F++ + Q LK +GLLHF E+LK+ N D +L V N +L+ F+++ Sbjct 553 IFNKTKGNPFYVKQFLKSVYDEGLLHFD FESLKWNFNSDLILQMNVS GNVVELMTSFIKK 612 Query 273 LPRKHIEKLLLLA-VQEKASKKTLNLL 298 LP +E L + + + + +K++L+L+ Sbjct 613 LPADKLELLKIASTIGTQFNKRSLSLI 639

MGF505-3R	40-192	Leucine-rich repeat serine/threonine- protein kinase 1 [Anoplophora glabripennis] (XP_018576821.1)	48	Query	40	LQRIEKN-QILVQQEPIHINEALKVAASEGNYEIVELLLSWEADPRYAVVGALESKYDDL	98
						LQ ++K+ ++++ P N L +A GN EIV++LL + AD RY V Y	
				Sbjct	169	LQSLQKDAELIINMAPSGANTLLFIACQIGNKEIVKILLQYGADGRYHSVTKYSPLYIAC	228
				Query	99	VYKYYDQVKDCHDILPLIQNPETFERCHELNSTC---SLKCLFKHAVINDMLP--ILQKY	153
						+ +++ V+ P + T ER +++ C + CL + N P +L+KY	
				Sbjct	229	YHGHHNIVELLLLKFPELVQQHTVERWLPIHACCINGHVSL--ELLFNFPYPTKVLRLKY	286
				Query	154	TDYLDRWEYCSQMLFELACSKKKYEMVWIEGVLGVGKV	192
						D ++WEY M F++ + +++ +LG K+	
				Sbjct	287	RDSTEQWEYF--MPFDINERDATGQNLLYVAALLGNKKL	323

Table 11-1 Alignments of BLASTp hits for MGFs of interest Hits include ascension number of proteins with highest score. Note – MGF360-11L, 14L, MGF505-4R, 5R did not return any results.