RECOGNITION OF HEPACIVIRUSES BY FICOLINS IN DIFFERENT MAMMALIAN SPECIES

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Student declaration:

I declare that the data presented is entirely my work, except where otherwise stated.

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Abstract

Ficolins are polymorphic liver-expressed pattern recognition receptors (PRRs) that contribute to the innate surveillance of virus infections, recognising carbohydrates such as N-acetyl glucosamine, which are components of glycoproteins found on the surface of different viruses. While human ficolin-2 has been demonstrated to bind and inhibit entry of hepatitis C virus (HCV) particles, the antiviral activity of ficolins found in other mammalian species is unknown. For the first time, recombinant mouse and non-human primate (NHP) ficolins were cloned, expressed and purified to determine their interaction with HCV and non-primate/Equine hepacivirus (NPHV/EqHV), and the impact on virus entry.

HCV and EqHV have been recently reported as closely related within the hepacivirus genera (family *Flaviviridae*). This relatedness may provide insights on the potential use of EqHV as a model for the study of HCV. Both viruses encode two glycoproteins (E1 and E2), which are found on the surface of the viral envelope. These glycoproteins facilitate the entry into the host and are targets for host immune recognition molecules like ficolins. HCV and EqHV pseudoparticles (pp) were used to determine the neutralising ability of ficolins from different species. An EqHVpp model system for the investigation of entry and neutralisation of EqHV infection was created and validated.

In this study, for the first time, the neutralisation of hepacivirus by non-human ficolins was assessed. These findings may provide better insight into the divergent evolution of these genes in mammals. It is possible that ficolins might serve as a future therapeutic anti-viral agent for hepaciviruses.

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Publication

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Abbreviations

AcBSA	Acetylated BSA
AP	Alkaline phosphatase
BCA	Bicinchoninic acid
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BSA	Bovine serum albumin
Ca^{2+}	Calcium ion
$CaCl_2$	Calcium chloride
CD81	Cluster of differentiation 81
cDNA	Complementary deoxyribonucleic acid
CLD	Collagen-like domain
CLDN1	Claudin-1
CRD	Carbohydrate recognition domain
CRP	C-reactive protein
C-terminal	Carboxy-terminal
CysNAc	*N*-acetylcysteine
DAA	Direct-acting Antiviral
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DTT	Dithiothreitol
EBOV	Ebola virus
EBOVpp	Ebola virus pseudoparticles

ECL	Enhanced chemiluminescent
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EphA2	Ephrin receptor A2
EqHV	Equine Hepacivirus
EqHVpp	Equine hepacivirus pseudoparticles
ER	Endoplasmic reticulum
FBG	Fibrinogen-like
FBS	Foetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GlcNAc	*N*-acetylglucosamine
GNA	*Galanthus nivalis* agglutinin
gp	Glycoprotein
H77	Representative genotype 1 HCV strain
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C Virus
HCVcc	Hepatitis C virus cell culture
HCVpp	Hepatitis C virus pseudoparticles
HEK	Human embryonic kidney
HIV-1	Human immunodeficiency virus type 1
HRP	Horse radish peroxidase
Huh	Human hepatoblastoma
HVR	Hypervariable region
IAV	Influenza A virus
ICTV	International Committee on Taxonomy of Viruses
ID_{50}	Half-maximal (50%) inhibitory dilution effect

IFN	Interferon
Ig	Immunoglobulins
IRES	Internal ribosomal entry site
JFH-1	Japanese Fulminant Hepatitis-1
LB	Lysogeny broth
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LVP	Lipo-viro-particle
mAb	Monoclonal antibody
MAC	Membrane attack complex
MASP	Mannose-binding lectin-associated serine protease
MBL	Mannose-binding lectin
MEGA	Molecular Evolutionary genetic tool
MHC	Major Histocompatibility complex
MLV	Murine leukaemia virus
MW	Molecular weight
MWCO	Molecular weight cut off
MY	Million years
nAb	Neutralising antibodies
NaCl	Sodium chloride
NANBH	Non-A, non-B hepatitis
NCBI	National Centre for Biotechnology Information
NEAA	Non-essential amino acids
NEB	New England Biolabs
NHP	Non-human primate
NK	Natural killer
NPHV	Non-primate hepacivirus

NPHVpp	Non-primate hepacivirus pseudoparticles
NS	Non-structural
N-terminal	Amino-terminal
OCLN	Occludin
OD	Optical density
Opti-MEM	Minimal Essential Medium
ORF	Open reading frame
p	Probability value
PAMPs	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PBST	PBS containing 0.05% Tween 20
PCR	Polymerase chain reaction
PEG-IFN-α	Pegylated interferon alpha
PEI	Polyethylenimine
pNPP	para-Nitrophenyl phosphate
PRR	Pattern recognition receptor
PVDF	Polyvinylidene diflouride
RAS	Resistant associated substitutions
RBV	Ribavirin
rER	Rough Endoplasmic reticulum
RLU	Relative luminescence unit
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Real Time-Polymerase Chain Reaction
SAA	Serum Amyloid A
SAO	Serum amyloid protein

SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel
SNPs	Single nucleotide polymorphisms
SP	Signal peptide
SR-BI	Scavenger receptor class B member 1
SRPs	Signal recognition particles
ssRNA	Single-stranded ribonucleic acid
SVR	Sustained virologic response
TBS	Tris-buffered saline
TBST	Tris-buffered saline containing 0.05% Tween 20
TfR1	Transferrin receptor 1
TGF	Transforming growth factor
UTR	Untranslated region
VLDL	Very low-density lipoprotein
VLP	Virus-like particle
VSV	Vesicular stomatitis virus

1 Background Review

1.1 Ficolins

Ficolins are soluble pattern recognition receptors (PRRs) that recognise carbohydrates and acetylated compounds on the surface of pathogens, referred to as pathogen-associated molecular patterns (PAMPs) [1]. They are structurally similar to mannose-binding lectin (MBL) and other collectins [2] but possess a fibrinogen-like domain (FBG) in place of the carbohydrate recognition domains (CRDs) of the collectins [3]. Ficolins were initially described as transforming growth factor (TGF)-1 binding on the porcine uterine membrane and were classified as pig ficolin-α and ficolin-β [4]. Ficolins comprise a collagen-like and a fibrinogen-like domain. The globular fibrinogen-like domain at the C-terminus is the functional domain which recognises PAMPs.

1.2 Types of ficolins

Several ficolins have been discovered in humans and other non-human species and have been classified into groups. Humans have 3 types of ficolins: 1) M-Ficolin/ficolin 1/FCN1 [5]; 2) L-ficolin/ficolin-2/FCN2 [6, 7]; and 3) H-Ficolin/ficolin 3/FCN3 [8]. Ficolins in non-human species are: Ficolin A and B (FCNA and FCNB) in mice and rodents [9], the aforementioned pig ficolins, and the horseshoe crab ficolin tachylectins 5A/5B (TL 5A/5B) [10]. Also, homologs of human ficolin genes *FCN1, FCN 2 and FCN3* have been discovered in non-human primates [11]. Ficolins provide rapid protection of a host against infectious microorganisms, as they circulate in blood or localised within organs. Because of interchain disulphide bonds formed by cysteines of the N-terminal region, all ficolins oligomerise to tetramers and hexamers [12]. Table 1-1 shows the expression, synthesis and sugar specificity of distinct types of ficolins.

1.3 **Ficolin Structure and Organisation**

1.3.1 Structure of ficolins

Ficolins are proteins that multimerise to form oligomers of 35-40 kDa subunits. Their structure comprises a collagen-like domain with Gly-Xaa-Yaa repeats of different lengths, an N-terminal domain that contains cysteine residues and a fibrinogen-like domain (FBG) at the C-terminus which is the carbohydrate recognition domain (as shown in Figure 1-1) [6, 13]. The collagen-like domain is used for recruiting MBL associated serine proteases (MASPs) proteins, which trigger the induction of an immune response, while the FBG domain is used for interaction with pathogen-associated carbohydrates (Figure 1-1) [6, 13]. Homotrimers are the functional unit of a ficolin, formed by the association of its single chains. Thereafter, multimers of trimeric subunits are formed by a further association of ficolin trimers. An oligomeric structure (dodecamer) of human ficolin-1 and human ficolin-2 comprises four homotrimer subunits giving a “bouquet” like appearance whereas human ficolin-3 is octadecameric [2, 6, 13, 14]. Recently, studies have shown that FCN3 is sialylated [15]. Murine Ficolin A (FCNA) also forms higher order oligomers, which are mostly tetramers comprising 12 subunits [16].

Table 1-1 ***Types of ficolins and their characteristics***

Species	Ficolin Type	Synthesis	Tissues of Expression	Sugar Specificity
Human	Human ficolin-2/L-ficolin (FCN2)	Liver	Serum	N-acetyl-D-Glucosamine (GlcNac), N-acetyl-D-galactosamine (GalcNac), N- acetyl-D- mannosamine (ManNac), N-acetyl-L—cysteine (CysNac), N-acetyl glycine (GlyNac), corticosteroids, elastin, acetylcholine, 1,3-β- D- glucan, and lipoteichoic acid.
	Human ficolin 1/M-ficolin (FCN1)	Monocytes	Monocyte surface and serum	N-acetyl-D-Glucosamine (GlcNac), N-acetyl-D-galactosamine (GalcNac) and Sialyl-N-acetyl lactosamine (SiaLacNac).
	Human ficolin 3/H-Ficolin (FCN3)	Liver and type II alveolar cells	Serum, bronchus, alveolus, bile	N-acetyl-D-Glucosamine (GlcNac), N-acetyl-D-galactosamine (GalcNac), glucose, fucose and polysaccharide.

Mouse	Ficolin A (FCNA)	Liver and spleen	Serum	N-acetyl-D-Glucosamine (GlcNac), N-acetyl-D-galactosamine (GalcNac)
	Ficolin B (FCNB)	Bone marrow and spleen	Peritoneal Macrophages	N-acetyl-D-Glucosamine (GlcNac), N-acetyl-D-galactosamine (GalcNac), Sialyl-N-acetyl lactosamine (SiaLacNac), Fetuin.

This table shows the main differences in terms of synthesis, tissue expression and sugar specificity for the well-classified mouse and human ficolin. All ficolins from different mammalian species share common structural and functional characteristics.

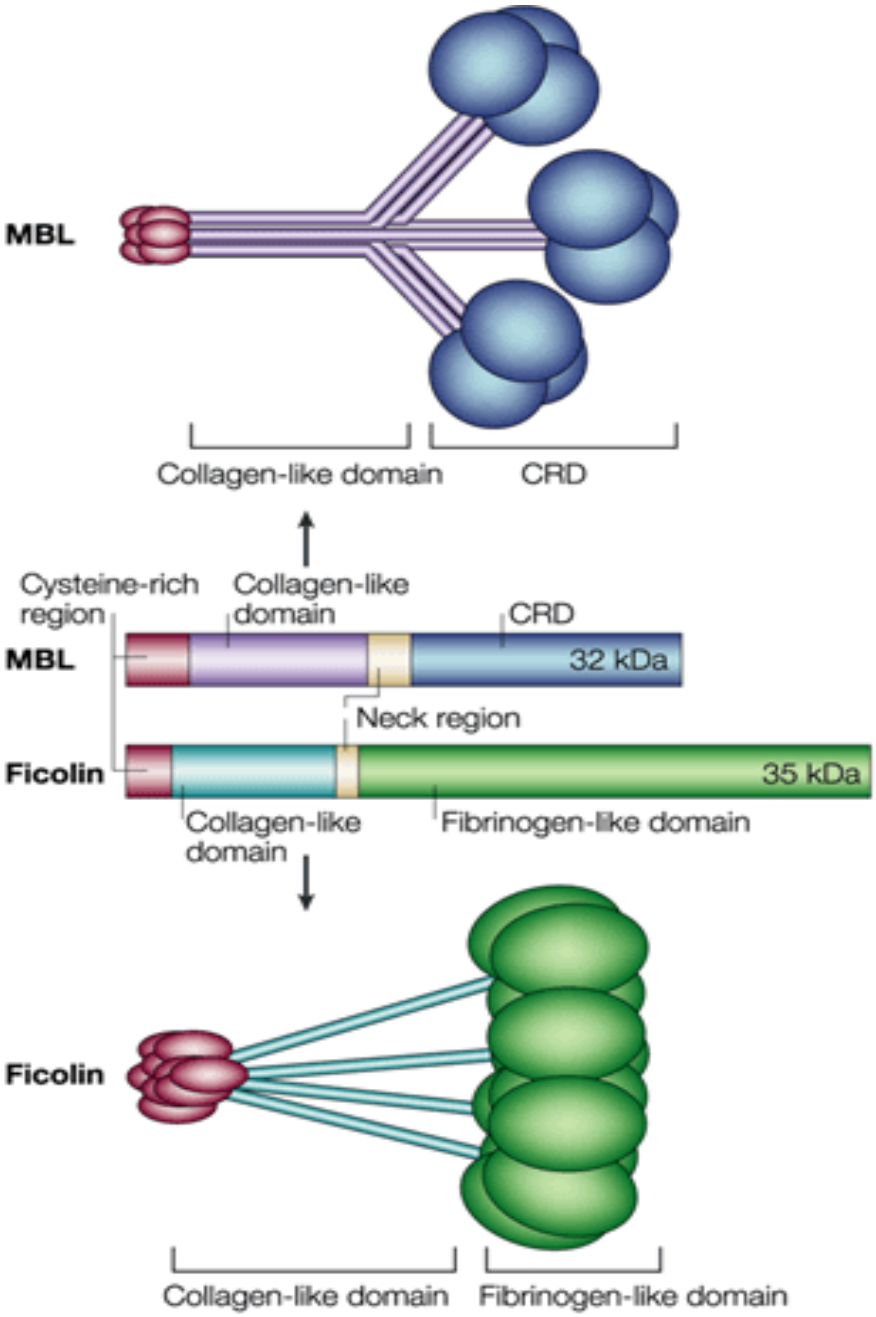

Figure 1-1 Structural organisation of MBL and ficolins

Structural organisation of MBL and ficolin. Both contain a short N-terminal cysteine-rich region followed by a collagen-like domain. For MBL, the C-terminal region is the binding domain (carbohydrate-recognition domain) shown as blue ovals, while ficolins possess a fibrinogen-like domain (green ovals), shown in tulip forms. Polypeptides interact through the collagen-like domain, forming triple helices (trimers) which leads to higher oligomeric forms (tetramers and hexamers). The lectin pathway of the complement is activated when the MASPs interact with the collagen-like domain.

1.3.2 Exon organisation of ficolin genes

Human ficolins 1, 2 and 3 are encoded by distinct genes, namely; *FCN1, FCN2* and *FCN3*. These encode polypeptides of 326, 313 and 299 amino acids, respectively, including the signal peptide [17]. *FCN1* and *FCN2* genes are positioned at chromosome 9q34 and *FCN3* at chromosome 1p36.11. *FCN1* gene possesses nine exons while both *FCN2* and *FCN3* genes comprise eight exons [18] as seen in Figure 1-2 a. The first exon encodes the signal peptide and the first nine N-terminal residues while the second and third exon encodes the collagen-like domain. The fourth exon encodes a short connecting region called a linker domain. Exons five to eight (or nine in *FCN1*) encode the fibrinogen like domain at the C-terminal. In mice, *fcna* and *fcnb* genes comprise ten and nine exons, respectively. The first exon, exon two to four, the fifth and sixth (in *fcna*) or the fifth (in *fcnb*) and the last four exons, encode a signal peptide, the collagen-like domain, the linker and the FBG domain respectively [19]. The exon organisation of mouse *fcnb* is like that of human *FCN1,* showing that the *fcnb* is the mouse orthologue (sequences which have a common ancestor and have split because of a speciation event) of human *FCN1* (Figure 1-2b).

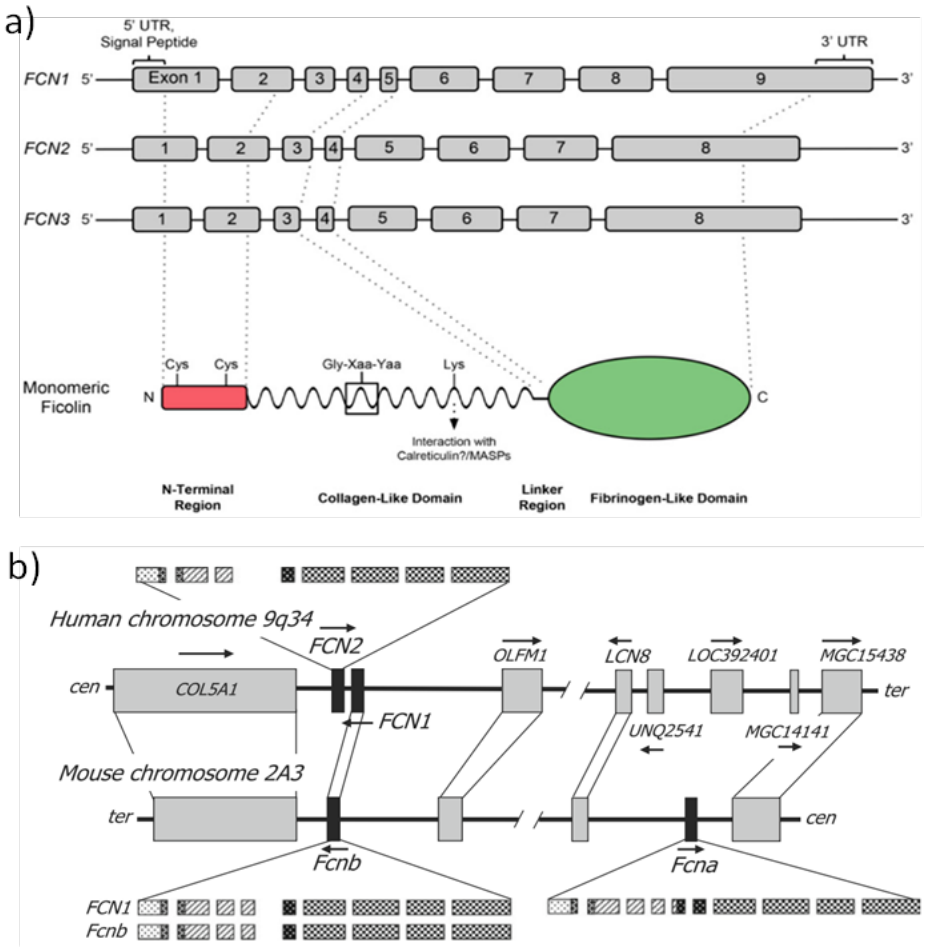

Figure 1-2: Exon organisation of ficolins genes

Exon organisation of human ficolin genes (a). *FCN1* gene comprises nine exons while *FCN2* and *FCN3* genes are made up of eight exons. The monomeric ficolin shows the exon number of each domain and other structural features. (b) compares human ficolin genes (*FCN1* and *FCN2*) and mouse ficolin genes (*fcna and fcnb*) on chromosomes 9q34 and 2A3 respectively. Human *FCN1* and mouse *fcnb* are similar in locus and exon-intron structures. In contrast, the exon-intron structures of *FCN2* and *fcna* differ from each other and the locus of *fcna* is far from the homologous region of *FCN2.* The exon organisation was referenced from [18] and [20].

1.4 **Ligand Binding properties of Ficolins**

The ligand-binding activity of ficolins is attributed to the FBG domain, which possesses multiple binding sites. The binding of ligands to the FBG domain allows ficolin to activate the lectin pathway of complement or act as an opsonin to enhance phagocytosis. All human ficolins bind to acetylated compounds including *N*-acetylglucosamine (GlcNac), *N*-acetylcysteine (CysNAc), *N*-acetylgalactosamine (GalNAc), and acetylated human serum albumin [21-25]. Ficolin 1 binds to sialic acids [26] while ficolin 3 binds to D-fucose but not mannose and lactose [12, 27]. Recombinant ficolin 1 was shown to form a complex with MASPs, resulting in complement activation upon binding to GlcNAc [25, 26]. Ficolin 2 is the best-characterised ficolin and binds to a wide range of microorganisms. Besides binding to sugars, ficolin 2 binds other substances such as lipoteichoic acid (LTA), N-glycans, hemagglutinin (HA), neuraminidase (NA) and 1,3-β-D-glucan [28-32].

The orthologues of human FCN2 and FCN1 in mice are ficolin A and B, which have different sites of synthesis and expression as shown in Table 1-1. The third ficolin (FCN3/mouse ficolin H) is a pseudo-gene in mammals other than humans [19]. Recombinant FCNA and FCNB bind to GlcNAc and GalNAc. Complement component 4 (C4) is activated by the association of recombinant FCNA with MASP-2 [33]. Although FCNB was initially thought not to bind to MASPs like other ficolins [33], further studies showed that a purified FCNB contained trace amounts of MASP-2 and exhibited C4 activity on GlcNAc-coated microplates [34]. It was also shown that FCNB recognises sialic acid, similar to human ficolin-1. Ficolins of non-human primate (NHP) origin have the same characteristic oligomeric structure as seen for proteins found in humans [11]. However, the binding specificity of NHP ficolins have not yet been studied. The

varied ligand specificity of the different types of ficolins is attributed to the different binding sites in their fibrinogen-like domains. To reveal the determinants of these specificities, the structure of the recombinant carbohydrate recognition domain of human ficolins has been solved by x-ray crystallography in complexes with different ligands [12, 35, 36]. The proteins were shown to be homotrimeric, homologous to a fibrinogen-like invertebrate lectin, tachylectin 5A (TL5A) [37], but showed different structures and specificities. The homotrimer formed in the crystallised structures is as expected because of the trimeric nature of ficolins [3]. Four binding sites in the human ficolin-2 FBG domain have been identified (S1-S4), which possess different binding specificities. An outer S1 binding site important in the recognition of GlcNAc for all ficolins was identified [12]. Likewise, the calcium ion (Ca^{2+}) binding site was found in a loop region that represents the most external part of the trimers (Figure 1-3).

The structure of human ficolin-2 has evolved to be a flexible protein able to recognise diverse carbohydrates and acetylated targets through different sites in a variety of ligands in both calcium dependent and independent manner [12]. GlcNAc, CysNAc and neutral galactose were found in the S2 site while various N-acetylated structures were in the S3 site. Cooperation of S3 and S4 sites were involved in the recognition of 1,3-*β*-D-glucan. Binding of ficolin-2 to some ligands has been reported to be calcium dependent [6, 38]. However, in contrast to MBL (a calcium-dependent lectin) binding [39, 40], the conserved calcium ions do not take part directly in ligand binding [41]. Thus, the response to ethylenediaminetetraacetic acid (EDTA) in some binding assays might be because of indirect effects related to the structural stabilisation of the S1 binding site close to the calcium ion. Further to this, human ficolin-2 was shown to bind to GlcNAc or CysNAc-Sepharose beads not only in the presence of calcium but

also in the absence of calcium at high sodium chloride (NaCl) concentrations in the presence of EDTA [38]. To some extent, this inconsistency can be explained by the binding sites being indirectly affected by their ionic environments. Many lectins interact with their ligands using a “lock-and-key” binding mode; with a slight change in their conformation upon binding [41]. Therefore, calcium ions organise and stabilise the FBG domain and its binding sites. It thus seems possible that the disruption of the polarity of the binding sites will provide ficolin with calcium-independent binding [15, 32, 42] under certain conditions. No structural insights into the recognition properties of non-human ficolins have been discovered so far. Hence, the well-established knowledge of human ficolins can better understand the ligand-binding specificity of ficolins from other species. The crystal structure of the human ficolin-2 and its binding sites are shown in Figure 1-3.

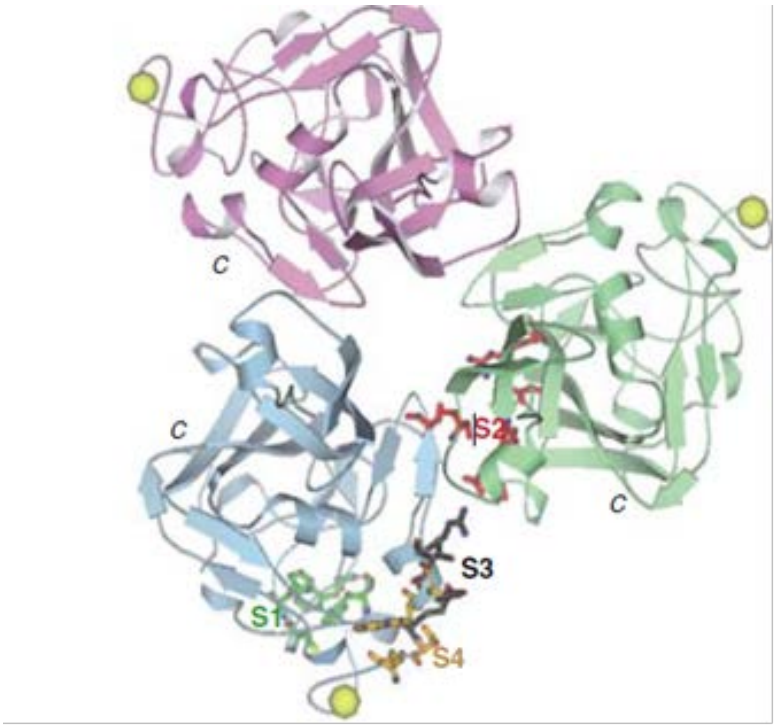

Figure 1-3 Homotrimeric structure of the recognition domain of human ficolin-2 (PDB ID: 2j3g) and their binding sites

The binding sites are displayed as ball and sticks and are coloured green, red, black and orange for S1-S4, respectively. Ca^{2+} ions are represented as golden spheres. This figure was adapted from Garlatti et al. [12].

1.5 Expression of Ficolins from different Mammalian Species

Human ficolins are expressed distinctively in different organs. Ficolin-1 mRNA is expressed in monocytes, lung and spleen [5, 7, 43]. The ficolin-1 protein is detected on membranes of monocytes and granulocytes [44, 45]. It is also present in serum [46] in a relatively low concentration of about 0.3 $\mu g.mL^{-1}$ [47]. Ficolin-2 is expressed in the liver and circulates in the blood [48] with a median serum concentration of 5 $\mu g.mL^{-1}$ [49]. FCN3 is the most abundant ficolin in serum with a median concentration of 25 $\mu g.mL^{-1}$ [50] and it is predominantly expressed in the liver and lungs [51]. In mice, FCNA is expressed in the spleen and liver [19] while FCNB is expressed in cells of myeloid cell lineage in the bone marrow [9, 16]. Human and mouse ficolins (FCN2, FCN3 and FCNA) are classified as plasma types. They are mainly produced in the liver. Non-plasma types (FCN1 and FCNB) are found at low level in plasma [20]. FCN2 and FCN3 have been detected in NHP sera with their concentrations determined [11]. It was hypothesised that the NHP ficolins are homologous to human ficolins. However, no NHP ficolins have been expressed from any organ. This study showed that NHP ficolin can be expressed in the liver as human ficolin.

1.6 Function of Ficolins

1.6.1 Ficolins as pattern recognition receptors (PRRs)

Ficolins are PRR molecules of the innate immune system which recognise and bind to carbohydrate moieties on the surface of the pathogen and abnormal self-cells. Recent studies have shown that human ficolin-2 binds to influenza virus and *Streptococcus pneumoniae,* whereas human ficolin-3 binds and inhibits

entry of influenza virus [15, 29, 52, 53]. Hence, the antiviral activities of ficolins as PRRs lead to the activation of complement and other immunological responses to inhibit viral action.

1.6.1.1 Complement activation

The complement system is a component of the innate immunity as it forms one of the first lines of defence against pathogens and priming adaptive immunity. It comprises greater than 60 components and activation fragments [54], which include recognition molecules with effector functions [38], nine central components of cascade (C1-C9), regulators, inhibitors and binding proteins. In previous studies, MBL was found to be a prototypic initiator of the lectin pathway. However, it is now well established that collectin 10 and 11 (CL10 & CL-11), and ficolins, utilise MASPs to activate the complement system [26, 55-57].

Complement activation occurs successively in four steps. The first step is the initiation of the complement activation pathway, which is induced by spontaneous activation on biological surfaces or the recognition of an antibody bound to an antigen or through binding of carbohydrate recognition complexes (e.g. MBL, ficolins) to carbohydrates on the surface of a pathogen. This activation occurs via three pathways: a) classical pathway b) alternative pathway, or c) lectin pathway (see Figure 1-4). The classical pathway is antibody dependent and is activated by antigen-antibody complexes. The alternative pathway is initiated by covalent binding of C3 to hydroxyl or amine groups on the cell surface of pathogens [54] showing it does not require any recognition molecule. The lectin pathway is activated by specific carbohydrate recognition subcomponents, which include; ficolins, MBL and CL-11 [52, 58, 59]. The second step of the complement activation is the cleavage of C4 and

C2 leading to the formation of C3 convertase (C4bC2b). This then cleaves C3 into C3a and C3b. C3a promotes the activation and recruitment of innate effector cells. The formation of C5 convertase is the third step, cleaving C5 into C5a and C5b. The latter starts with the terminal step whereas C5a is an effective anaphylactic peptide that induces inflammation. C5b assembles the terminal pathway components; C5, C6, C7, C8 and C9 eventually start the formation of the membrane attack complex ensuring cell lysis and death of target cells (Figure 1-4) [54, 60].

1.6.1.2 The lectin pathway of the complement system

The lectin pathway of the complement system is activated via the recognition of PAMPs by PRRs. Similar to the classical pathway, except that it is activated by PRRs that could either be ficolins, MBL or other collectins such as CL-11. MBL and CL-11, recognize carbohydrate residues on the surface of different microorganisms, while ficolins primarily recognize and drive lectin pathway activation on acetylated ligand structures. This pathway can eliminate pathogens by complement-mediated lysis, opsonisation in the absence of antibodies, phagocytosis and apoptosis [60-62]. It is triggered by complex formations between MBL or ficolins and MASPs. The complexes are formed after binding of specific PAMPs, which catalyses the activation of MASPs; MASP-1, MASP-2, MASP-3. MASP-2 is the most essential effector enzyme of the lectin pathway as the general triggering of MASPs leads to the triggering of the downstream reaction cascade. MASP-2 starts the formation of C3 convertase (C4bC2b formerly C4b2a) by cleaving C4 and C2 and triggering their activation into their subcomponents: C4a, C4b, C2a and C2b forming the C3 convertase. MASP-2 cleavage is more efficient than the serine protease C1s but has a similar modular structure as both C1r and C1s (classical pathway

serine proteases). It can, therefore, activate the lectin pathway with or without other serine proteases [59, 63-65].

Unlike MASP-2, the mechanism of action of MASP-1 and MAP-3 is not well understood. While MASP-2 can activate the lectin pathway independently, MASP-1 can only cleave C4b bound to C2 but does not cleave C4 [66, 67]. MASPs circulate in the blood complexed with MBL and ficolins and also bind either of the proteins through their collagenous domain [26, 57]. MBL and ficolins have been shown to function independently of one another as they activate the lectin complement pathway [68].

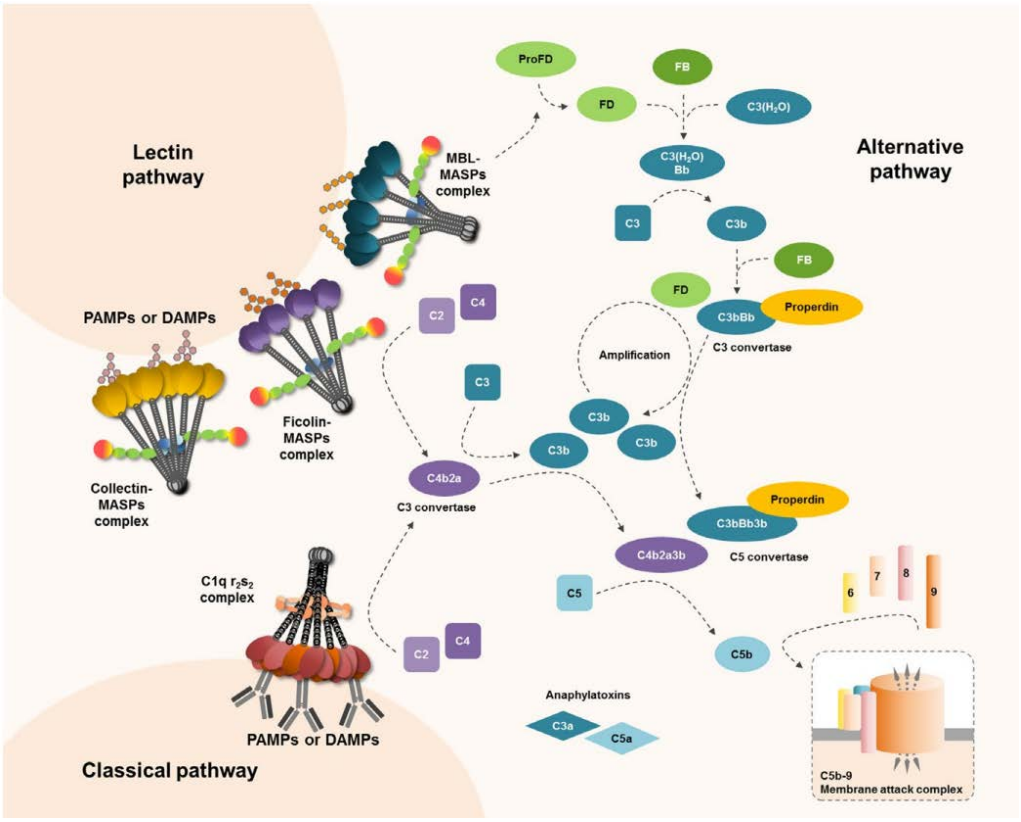

Figure 1-4: Overview of the complement system

The complement system comprises three activation pathways; the classical, the lectin, and the alternative pathway. The classical pathway is initiated by C1q binding directly to PAMPs or damage-associated molecular patterns (DAMPs), or indirectly via immunoglobulins. The lectin pathway is initiated by MBL, ficolins or other collectins such as CL-10, and CL11 binding to PAMPs or DAMPs. In the lectin pathway, PRRs are found in complexes with the MASPs, whereas C1q is found in complex with the serine proteases C1r and C1s in the classical pathway. Activation of the classical and lectin pathway leads to cleavage of C4 and C2 and the formation of a C3 convertase (C4b2a). The alternative pathway is activated by spontaneous hydrolysis of C3. Activation of C3 allows factor B (FB) to bind to C3. FB is then cleaved by factor D (FD) leading to the formation of the alternative C3 convertase (C3bBb). The alternative C3 convertase is stabilized by the plasma protein properdin. The C3 convertases cleave C3 into the opsonin C3b and the anaphylatoxin C3a. Activation of C3 also leads to the formation of the classical and lectin pathway C5 convertase (C4b2a3b) and the alternative C5 convertase (C3bBb3b). The C5 convertase cleaves C5 into the anaphylatoxins C5a and C5b, leading to the formation of a terminal membrane attack complex (C5b-9). The alternative pathway also works as an amplification loop for the classical and lectin pathways. The figure was adapted from Garred, P., et al., 2016 [69].

1.6.2 Ficolins can function as restriction factors

Restriction factors are antiviral proteins produced by the host that inhibit the replication of viruses during their life cycle in host cells, and they are part of the innate immune system. Restriction factors are a broader part of the innate

immune system that mediates cell-intrinsic immune response. Restriction factors have antiviral activity and can be induced by the action of interferons (IFNs) or directly by viral infection. Their antiviral action results in selection pressure on pathogens, leading to positive selection, and an arms race for adaptation of host and pathogen [70]. Hence, understanding the roles of ficolins as restriction factors in innate immunity is crucial. This will be interesting as ficolin genes in non-primate species have been investigated and polymorphisms observed, similar to the variation observed in humans [11]. During the evolution of mammals, the FBG domains of ficolin genes may be modified and might have co-evolved with pathogens leading to a greater activity against different pathogens [11]. SAMHD1 is a restriction factor that has been shown to have important roles in innate immunity through primate evolution and could serve as targets for therapeutic treatments [71]. Therefore, the knowledge of evolutionary traits of the NHP ficolin genes that will be discovered might provide insights into their activity as a restriction factor with antiviral activities when compared to human ficolins. This may give rise to a novel therapeutic treatment of different viral infections.

1.7 **Polymorphisms in Ficolin Genes**

All human ficolin genes are polymorphic [72, 73]. However, more significant single nucleotide polymorphisms (SNPs)-sites of substitution of a single nucleotide at a specific position in the genome/population-have been identified in the human *FCN2* gene. The distribution of SNPs has been found to differ between ethnicities [73, 74], which might have arisen from distinct geographical pressures. Polymorphisms in the *FCN1* gene have been associated with human FCN1 levels, with a mutation at -144C>A resulting in increased levels and the +7895TC mutation resulting in an inability to produce human FCN1 [75]. Two

other non-synonymous mutations (+6658G>A and +7959A>G) were also found to be associated with low human ficolin-1 levels, poor ligand binding ability, and low binding to group B streptococcus [75]. SNPs that were found to affect the structure and oligomerisation of human *FCN1* gene were; Gly43Asp, Arg93Gln and Trp279STOP [73]. In the human *FCN3* gene, only Leu117fs and Thr125Ala corresponding to the human *FCN2* gene Thr13Met and Val287Ala were predicted to affect human ficolin-3 function [73]. Polymorphisms in human ficolin-2 are the most widely reported and have contributed to disease outcome in bacterial [76-78] and viral infections [79, 80]. Three promoter SNPs (-986>G, -606G>A and -4A>G) in the human *FCN2* gene affect serum level differences including gene regulation and protein concentration [72]. SNPs in exon 8; +6359C>T (Thr236Met) and +6424G>T (Ala258Ser) in the FBG domain affects protein modification, folding and stability which alters protein function and binding to GlcNAc capacity [73, 74, 81]. The Arg147Gln and Arg157Gln mutations were also found to affect ligand binding, and these were found in the S2 and S3 binding sites respectively [73]. Arg103Cys and Thr137Met mutations were found to affect the chemical and structural properties of human ficolin-2. The amino acid changes clustered in exon 8 (which encodes the FBG domain) leads to variation in human ficolin-2 concentration and binding affinity. This may lead to some changes in host-pathogen interactions similar to that observed for MBL [82, 83].

1.8 **Evolution of Ficolin Genes**

A representative phylogenetic tree was constructed to further understand the evolutionary relationships of ficolin genes from different species (Figure 1-5). This tree suggests that the ficolin genes of each species have been duplicated independently. Alignment of the amino acid sequence of ficolins from different

species showed that mouse and rat ficolin-A, primate ficolin-2 and pig ficolin-α resulted from independent gene duplications and are different proteins (Figure 1-5) [17]. This implies that although these proteins are different, they have similar structural homology with human ficolins, and may have diverse recognition for viruses.

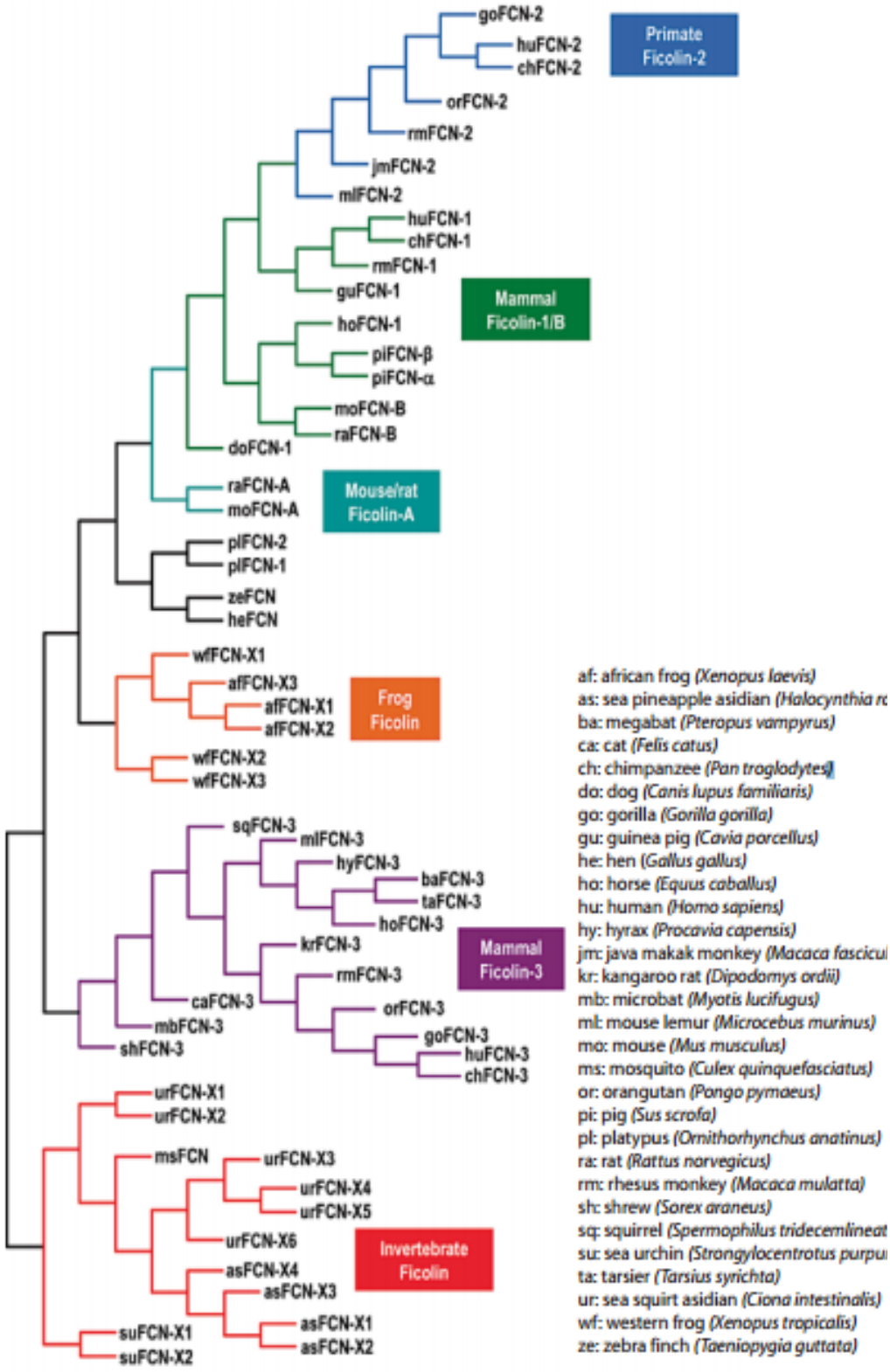

Figure 1-5: Phylogenetic neighbour-joining tree of the ficolin protein family

The tree was constructed based on alignments of the entire amino acid sequence of the ficolins using ClustalW [17].

1.9 **Interaction of Ficolins with Viruses**

Ficolins recognise glycans at the pathogen surface through their FBG domain. There have been several studies showing the inhibition or the enhancement of a viral infection by different human ficolins. A mouse model of influenza A virus (IAV) infection has been protected from the virus when exogenous human ficolin-2 and ficolin A were administered [29]. IAV has two surface glycoproteins (hemagglutinin (HA) and neuraminidase (NA)), which bind and interact with human ficolins. Following recognition, human ficolin-3 recognises and inhibits the infectivity of IAV strains to levels comparable to human ficolin-1 and ficolin-2 [15]. In the same study, binding and inhibition by human ficolin-3 were increased in the absence of calcium, which differs from findings with human ficolin-1 and pentraxin (PTX3) (a soluble PRR) where absence of calcium did not alter anti-IAV activity [84] or was calcium-dependent [85]. Conversely, all the human ficolins can interact with PTX3 to activate complement [81] or inhibit HA activity and infectivity of IAV [15]. In addition, different chimeras possessing parts of human MBL and ficolin-2, called recombinant chimeric lectin (RCL), were made, and these had a more potent antiviral effect when compared to the use of either of them. N-terminal domains of human ficolin-2 and CRD of MBL chimeric protein inhibit both IAV and Ebola virus and also the RCL could bind to Hendra, Nipah and Ebola viruses [86, 87].

Hepatitis C virus (HCV) possesses two highly glycosylated envelope proteins E1 and E2, which have been shown to bind to human ficolin-2. This interaction inhibits infection. However, high human ficolin-2 concentration was observed in the serum of individuals chronically infected with HCV, which led to the activation of the lectin pathway and a rapid antiviral response and inflammation of the liver [88]. Further to this, human ficolin-2 was discovered to inhibit HCV

entry at an early stage regardless of the infecting genotype [89]. A recombinant oligomeric form of human ficolin-2 was found to neutralise the entry of HCV virus in a dose-dependent manner [90]. It was also observed that calcium was important to access the binding site of human ficolin-2 during HCV neutralisation [90]. Results from the aforementioned study showed the antiviral activity of oligomeric recombinant human ficolin-2 as a PRR, inhibiting the entry of HCV and activating the complement cascade. This was a key advancement as the monomeric form of human ficolin-2 was previously described to activate the complement cascade but not inhibit HCV entry [31].

Human ficolin-2 acts as a PRR by recognising and binding to human immunodeficiency virus (HIV) through the N-glycans of its envelope glycoprotein (gp120). Subsequently, the lectin complement pathway was activated. In addition, the porcine plasma ficolin had been shown to reduce the infectivity of porcine reproductive and respiratory syndrome virus (PRRSV) [91]. The antiviral effect of human ficolins has been illustrated with the examples of viruses stated above, and this has yielded a suggestion of antiviral therapies. But nothing is known about the recognition and specificity of ficolins from different mammalian species. Also, there are no studies investigating viral recognition by NHP or mouse ficolins. Therefore, an improved understanding of this recognition might give insight to the virus-host interaction. This study will focus on the interaction of two viruses from the *hepacivirus* genus; Hepatitis C virus and non-primate hepacivirus.

1.10 **Hepaciviruses**

Hepacivirus is a genus in the virus family *Flaviviridae,* which comprises several positive-sense, single-stranded RNA viruses. For a very long time, HCV and GB-virus B (GBV-B) have been the only known members of the *Hepacivirus*

genus [92-94]. Recent members of this genus have been identified as non-primate hepaciviruses (NPHV), now called equine hepacivirus (EqHV) was firstly discovered in dogs and initially named canine hepacivirus (CHV) [95]. However, further studies showed that horses are the natural host of NPHV/EqHV [96-98]. Subsequently, hepaciviruses were discovered in other host species; bats (BHV) [99] rodents (RHV) [100] and Old World primates (GHV) [101], see Figure 1-6. HCV is the most investigated virus in the *hepacivirus* genus, but the discovery of EqHV could provide an additional tool to investigate the natural history and pathogenesis of hepaciviruses and could serve as a model for HCV.

In the past few years, a new taxonomy has been proposed for the *Hepacivirus* genus [102]. This has been accepted and updated by the International Committee on Taxonomy of Viruses (ICTV) [103]. This taxonomy places hepaciviruses that infect humans in species C while that infecting horses were designated in species A. Hence HCV and its divergent genotypes are placed in *Hepacivirus C* as members of the same species [102] while EqHV is classified as *Hepacivirus A* (Figure 1-6).

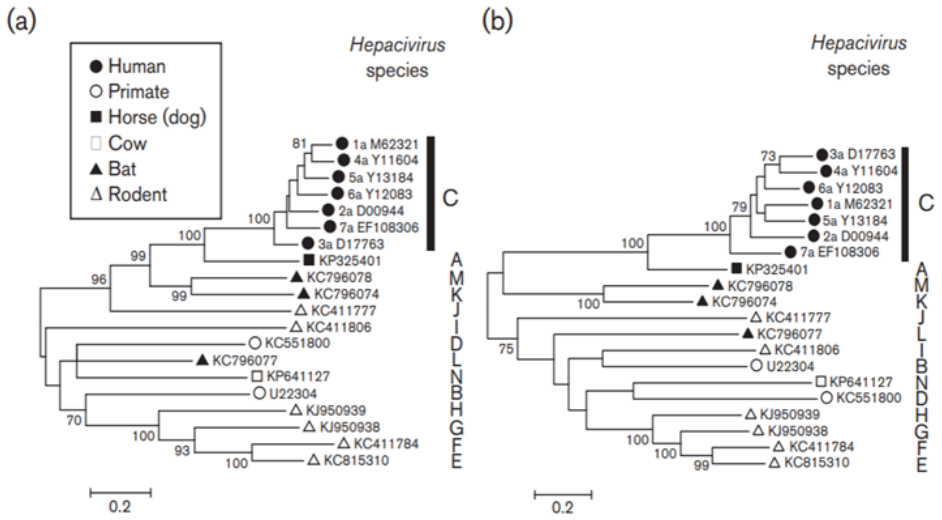

Figure 1-6: Maximum likelihood phylogenetic tree of hepaciviruses

The conserved regions of the hepacivirus polyprotein at positions 1123-1566 (a) and 2536-2959 (b) were analysed by constructing a phylogenetic tree using the maximum-likelihood method. All identified hepacivirus species were indicated with letters A-N as their proposed hepacivirus classification [102]. Hepatitis C is classified as hepacivirus C, and NPHV/EqHV is classified as hepacivirus A.

1.11 **Hepatitis C virus (HCV)**

1.11.1 Overview

HCV is estimated to affect 71 million people in the world, which is approximately 2% of the world's population [104-106]. It is a complex disease which causes acute and chronic infection and escalates into liver cirrhosis and hepatocellular carcinoma (HCC) [107]. More knowledge about cellular and structural biology of HCV, and its life cycle [108, 109], has led to the development of new directly acting antiviral (DAA) compounds to treat the disease [110]. However, a proportion of patients (1-15%) with chronic hepatitis C failed DAA based therapy [111, 112]. Viral variants harbouring resistance-associated substitutions (RAS) are associated with the failure of DAA, especially in NS5A [112, 113]. Second-generation DAA combinations can achieve sustained virological responses

(SVR) in the presence of RAS in variants harbouring them despite their persistence [114]. However, these combinations are costly and not widely available [115]. Hence an effective therapeutic vaccine is needed which will lead to a global eradication and elimination of HCV infection. HCV entry is a target for this intervention as immunotherapy has been proposed. As entry has been shown to be inhibited by ficolins, they could become a second-line therapy.

Recently, NPHV/EqHV has been found to be the closest relative of HCV [95] and studying this virus might improve our understanding of HCV. The HCV E1/E2 envelope glycoprotein complex is highly glycosylated and has been proposed to be essential for HCV entry [116, 117]. Both proteins possess exposed regions that are targets for host immune recognition [118].

1.11.2 Discovery and structure

HCV was first described as a non-A, non-B hepatitis (NANBH), a major cause of post-transfusion hepatitis [119, 120]. In 1991, the genome was discovered to have similar organisation and sequence homology with flaviviruses and pegiviruses [121]. HCV is an enveloped positive-strand RNA virus of the *Flaviviridae* family and genus *Hepacivirus* [122]. HCV possesses a single-stranded RNA genome, 9.6 Kb in size, encoding a single open reading frame (ORF) polyprotein precursor of approximately 3000 amino acids flanked by conserved and highly structured 5' and 3' non-translated regions (NTRs) [123] required for replication and RNA translation [109]. Within the 5' untranslated region (UTR) is an internal ribosome entry site (IRES) responsible for cap-independent translation. Host signal peptidases cleave the structural proteins; core, E1 and E2 [124, 125]. HCV envelope glycoproteins E1 and E2 are transmembrane proteins anchored in the viral envelope and are responsible for virus entry. P7 separates the structural proteins (core and the envelope

glycoproteins) and the non-structural proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B, that are essential for the viral genome replication [126, 127]. The proteins are encoded in the HCV genome and its organisation is shown in Figure 1-7.

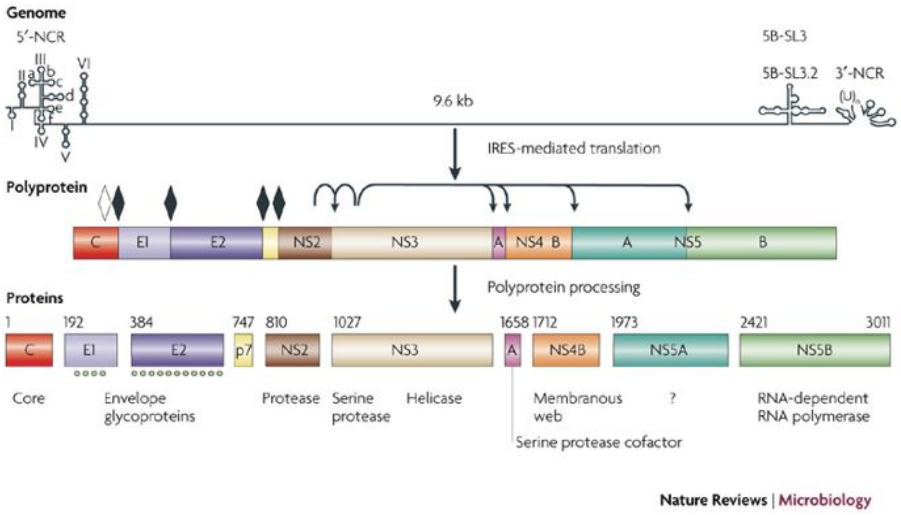

Figure 1-7: Genomic Structure of Hepatitis C virus

Genomic architecture of hepatitis C virus, highlighting the structural genes expressing proteins incorporated into virus particles and the non-structural genes that encode enzymes and co-factors essential for genome replication and assembly of virus particles [109].

The glycoproteins E1 and E2 are transmembrane proteins anchored to the host-derived double layer lipid envelope. Within the envelope is the nucleocapsid which is composed of the viral genomic RNA and the capsid protein (core) [128]. The nucleocapsid is spherical and approximately 30 nm in diameter. Electron microscopy has identified that infectious HCV virions are 40-100 nm size [129]. The virions circulate as complexes with very-low-density lipoproteins (VLDLs) and low-density lipoproteins (LDLs) as lipo-viral particles (LVPs) [129]. These lipo-viral particles have also been observed in serum and in cell culture-derived HCV [130-132].

1.11.3 Transmission, epidemiology and classification of HCV

HCV affects approximately 2% of the world's population [133]. Some individuals might have cleared the virus because of treatment or resolved spontaneously, hence reducing the viraemic prevalence [134]. The availability of global data is limited, and HCV continues to be a major global health problem. HCV is a blood-borne virus transmitted principally by needle sharing in intravenous drug users (IVDU) or trauma resulting from inadequately sterilised medical instruments, transfusion of blood and blood products and less often by sexual exposure or vertically from mother to child. HCV is classified into 8 major genotypes (1-8) based on their nucleotide sequence [135, 136]. The defined genotypes differ from each other at over 25% of nucleotide sites across the genome. The geographical region is an important determinant of the diversity and prevalence of HCV genotypes, as the virus has adapted in different host population backgrounds. Genotypes, 1a, 1b and 3a are predominant across Europe while genotypes that exist in Africa and Asia are more diverse. In most of Europe and the United States, chronic liver disease and cirrhosis are commonly caused by chronic HCV [137] and this has been attributed to the emergence of hepatocellular carcinoma (HCC) as a risk factor.

1.11.4 HCV envelope glycoproteins and functions

The two envelope glycoproteins E1 and E2 are type I transmembrane glycoproteins and are released from the polyprotein by signal peptidase cleavage. The glycoproteins E1 (192 aa; 35 kDa) and E2 (363 aa; 72 kDa) are assembled to form non-covalent heterodimer (E1/E2) [138, 139]. They are retained in the endoplasmic reticulum and found as large oligomers on the surfaces of HCV particles [116, 140]. E1 is reported to be involved in virus-cell membrane fusion, while E2 initiates viral attachment [141]. Therefore, expressing these proteins together enables them to form correctly folded proteins [142] and the production of infectious virus particles [143, 144].

1.11.4.1 Glycosylation of E1 and E2 glycoproteins

The HCV envelope glycoproteins are highly glycosylated. E1 has been shown generally to have five N-glycosylated sites while E2 has, on average, eleven N-glycosylated sites that are modified with glycans [145] as depicted in Figure 1-8. Four glycosylation sites at E1 and nine sites at E2 are highly conserved, despite the genetic variability exhibited by these glycoproteins [146]. Using retroviral particles pseudo-typed with genotype 1a HCV glycoproteins (HCVpp), studies have confirmed the importance of these glycans and their major functions in HCV which are; protein folding, replication, protection against neutralisation and entry [145-147]. The mutations at different glycosylation sites have different effects on HCVpp infectivity. Interestingly, glycans at positions E2N1, E2N6, and E2N11 contributed to HCV evasion of the humoral immune response by reducing the reactivity of HCVpp to the CD81 co-receptor to its binding site on E2. Therefore, targeting glycans on the surface of HCV and other viruses with highly glycosylated envelope proteins can be inhibited by carbohydrate-binding agents such as cyanovirin-N [148], or carbohydrate-binding recognition molecules such as ficolins and mannose-binding lectin [15, 87, 90, 149]. Targeting glycans of glycoproteins can be a novel antiviral therapy [150].

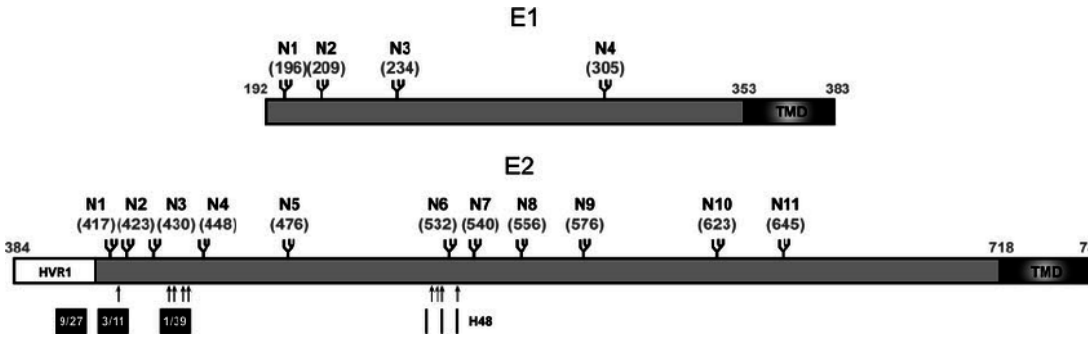

Figure 1-8: N-glycosylation sites in HCV glycoproteins E1 and E2

Positions of N-linked glycans are indicated with a branched structure. The positions are named with an N followed by a number indicating the glycosylation site within the sequence. The positions of the glycosylation sites in the polyprotein of the reference strain H (GenBank accession no. AF009606) are shown in parentheses. Epitopes recognised by monoclonal antibodies, 9/27, 3/11, and 1/39 are shown in dark boxes. TMD stands for transmembrane domain. Adapted from Helle, F., et al. [151].

1.11.4.2 The role of E1 and E2 glycoproteins in HCV entry

The E1 and E2 glycoproteins are involved in distinct steps of the HCV life cycle, including the production of infectious viral progeny, evasion of host immune recognition and facilitating entry of virions into the target cells [141, 152, 153]. HCV entry is a complex process that involves various cellular proteins and the envelope glycoprotein complex E1/E2 which is the obvious candidate ligand for host cellular proteins as it is present on the surface of the virion. E1 and E2 mediate viral entry by interacting at least with four essential cellular receptors; human cluster of differentiation 81 (CD81), scavenger receptor class B type 1 (SR-B1), tight junction proteins claudin-1 (CLDN1) and occludin (OCLN) [154-156]. Other host factors such as the receptor tyrosine kinases, epidermal growth factor receptor (EGFR) and ephrin receptor A2 (EphA2) [157], the cholesterol transporter Niemann-Pick C1-Like 1 (NPC1L1) [158], transferrin receptor 1 (TfR1) [159] and others [160] have also been implicated in the HCV entry process. CD81 and SR-B1 were identified by their ability to bind soluble and recombinant HCV E2, respectively [155, 161]. Although HCV glycoproteins interact directly with SR-B1 and CD81, interactions with CLDN1 and OCLN are not well defined [160], there are evidence-based studies on their role in entry.

The mechanism of HCV entry into hepatocytes involves lipo-viral particles captured by the interaction with apolipoproteins [162], enabling the interaction with low-density lipoprotein (LDL) that interacts with SR-B1, which normally mediates lipid metabolism as a high-affinity lipid receptor [163], exposing binding sites in the E2 glycoprotein which subsequently allows the binding of viral particles to CD81 [155]. Blocking interactions of SR-B1 during HCV entry have been shown to inhibit entry of HCV cell culture (HCVcc) particles of different densities, which represent different types of LVP [164]. The interaction between E2 and CD81 induces E1/E2 conformational

rearrangement, priming HCV internalisation and low pH-dependent fusion [165]. Eventually, the virus is taken up into the cytosol through clathrin-mediated endocytosis and the viral genome is released in a pH-dependent manner. Once inside the hepatocytes, the virus infects the cell. HCV is capable of cell-to-cell interaction *in vitro* by using hepatoma cells such as Huh-7.5 cells [166].

1.11.5 HCV life cycle

The HCV life cycle occurs through a series of stages; attachment and entry, translation and replication, budding and release (Figure 1-9). The envelope glycoproteins mediate the binding of the virus to the host, resulting in internalization through endocytosis. Viral entry is achieved through interaction with co-receptors starting receptor-mediated endocytosis and a pH-dependent fusion as reviewed in section 1.11.4.2. Viral RNA is released into the cytosol after the viral genome is liberated from the nucleocapsid (uncoating) [167]. The translation of the positive-sense RNA occurs through the internal ribosome entry site (IRES) in the cytoplasm at the rough endoplasmic reticulum (rER) in a cap-independent manner. The IRES facilitate the interaction of the viral gnome with host cell translation complexes [168]. The polyprotein translated from the genome is cleaved by host and viral proteases in the ER. To replicate the viral genome, HCV induces the formation of a membranous web [169] which is the site of replication complexes. This comprises NS3-4A, NS4B, NS5A and NS5B and are crucial for HCV replication. The complexes also generate negative-sense anti genomes which function as a template to produce positive-sense genomes as depicted in Figure 1-9. However, the negative-sense antigenomes are produced in low quantities compared to the positive-sense genomes [170].

The last stage of the HCV life cycle is viral assembly and release. Although the process is not well understood, it requires the core, E1, E2, p7 and the HCV genome to be brought together and packaged to produce infectious virions. Particles are released from the cell by exocytosis by passing through the secretory pathway [171].

The particles acquire low-density lipoproteins and the glycoproteins contain complex glycans by undergoing post-translational modification [140].

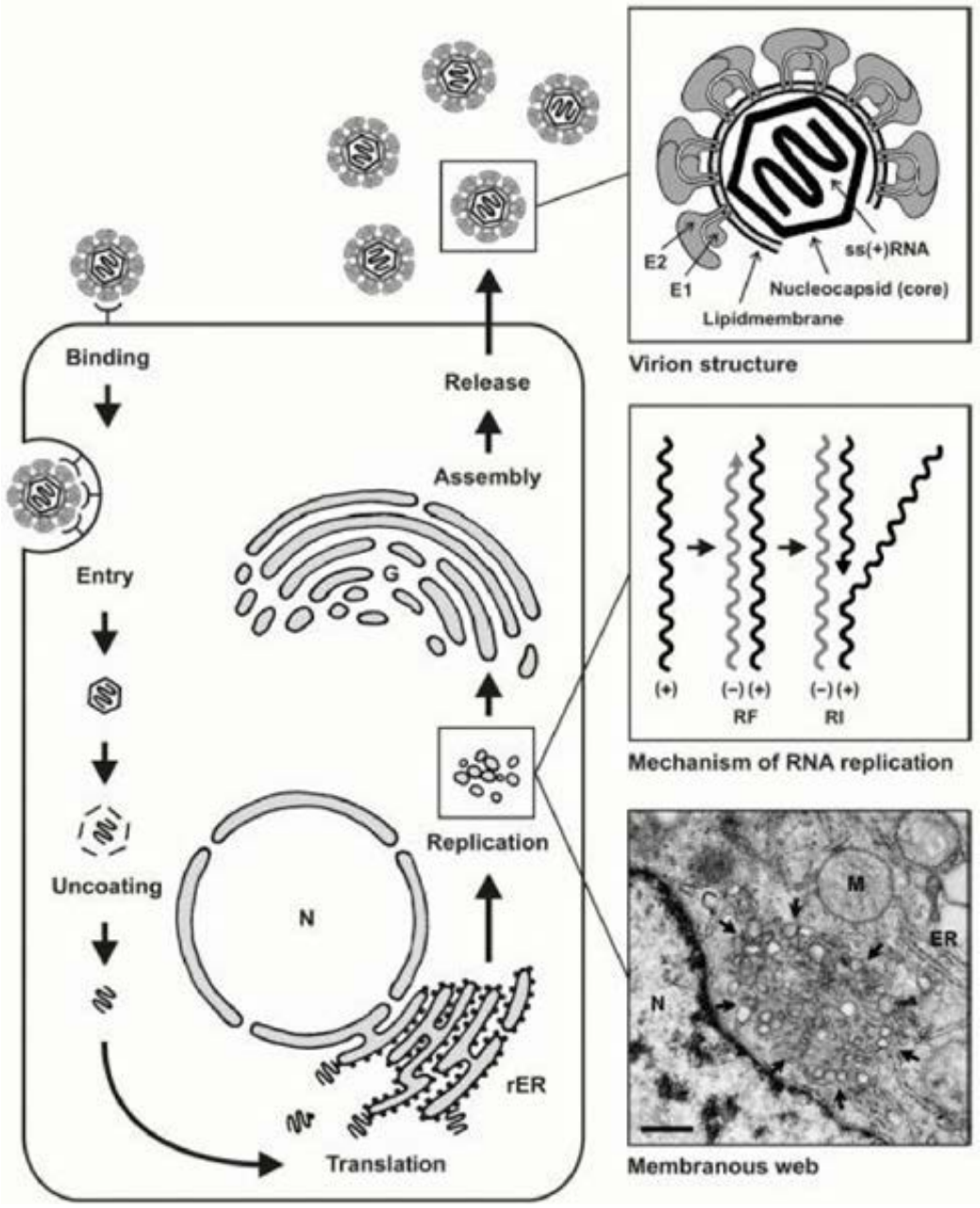

Figure 1-9: HCV Life Cycle:

HCV virions bind to the host cell and are internalised through interaction with host co-receptors. Translation occurs on the endoplasmic reticulum (ER) after uncoating of the viral genome. HCV proteins induce a membranous web which is believed to be the replication machinery. A model of HCV genome replication with a double-stranded replicative form as RF and that of a relative intermediate as RI. Progeny virions are assembled in the ER and are released [123].

1.11.6 Immune response in HCV infection

1.11.6.1 Role of innate immunity in HCV Infection

HCV infection results in the induction of both innate and adaptive immune responses. The innate immune response to viral infection represents the first line of defence against HCV and it triggers antiviral immune responses [172, 173]. This leads to opsonisation, inflammation, modulation of adaptive immunity [174-177] as well as the surveillance of organs and their circulation and direct neutralisation of the infection [149, 178, 179]. A complex interplay occurs between the humoral and cellular components of innate immunity. These cellular components include; natural killer (NK) cells and NK-T cells, monocytes, dendritic cells and platelets. NK cells suppress HCV replication in hepatic cells in response to HCV infection by mediating unrestricted major histocompatibility complex (MHC) cytolysis of virus-infected cells [180, 181]. Innate humoral immunity is mediated through germ-line encoded receptors (PRRs) [182]. PRRs recognise PAMPs presented on the surface of infected cells or on the infecting virus particles. They are produced by different cell types, including; lymphocytes, monocytes and hepatocytes. Complement components, MBL, ficolin-2, ficolin-3 and mouse ficolin A are produced in hepatocytes.

The initiation of an adaptive immune response to HCV infection depends on a successful acute phase innate immune response [183]. Inflammatory response and presentation of viral antigens to T cells are initiated when microbial PAMPs such as HCV glycoproteins bind to antigen-presenting cells [184-187]. This initiation and activation produce soluble cytokines, interferon (IFN) (IFN-α/β), interleukin (IL) (IL-1α), IL-1β and TNF-α and the IL-6 family of cytokines leading to the stimulation of the hepatocytes to produce acute-phase proteins (Figure 1-10) [183]. Interferon may induce the expression and presentation of HCV antigens on the infected hepatocyte surface, which stimulates the generation of immunoproteasomes [188]. The acute phase proteins are classified as type I and type II as reviewed by Han Moshage [189].

Type I proteins include complement components such as C-reactive protein (CRP) are induced by the action of IL-1 α, IL-1 β and TNF α while type II proteins are induced by IL-6 produced from macrophages. Some of these proteins have a role in pathogen recognition and combine with circulating PRRs (such as the defence collagens) to recognise pathogens and recruit cells for activating adaptive immunity (Figure 1-10).

1.11.6.2 Immune response Evasion

HCV infection induces inflammatory responses [190]. To persist in the presence of an immune response, HCV uses multiple mechanisms to evade the innate immune recognition, including elements of the humoral innate immune system such as the complement components; C4 and C5-9 [191, 192], MBL [149], ficolins [193], C1q-R and serum amyloid A (SAA) [132, 194]. HCV glycoproteins mediate the association of HCV virion and lipoproteins circulating in the blood [131]. Ultimately, this association forms lipo-viral particles [132] and leads to the resistance to antibody-mediated neutralisation [195, 196]. Also, evasion of neutralising antibodies targeting the envelope glycoproteins is mediated by the extensive glycosylation of the HCV virion, which reduces their immunogenicity [147, 151]. This notwithstanding, the targets for innate immunity continue to exist as glycosylation motifs serve as targets for defence collagens (such as ficolin and MBL) and they are essential for HCV biogenesis [183]. Therefore, the neutralising effect of innate binding proteins on virus replication or entry might lead to the preferential selection of mutants that can escape this recognition. The patterns of envelope glycoprotein glycosylation sites vary between isolates, suggesting that the glycosylation surrounding the virus particles is flexible [117]. Studies of Influenza A (H1N1) pandemic viruses confirmed the proposition that the defence collagens precisely apply selective pressure on a virus population [197]. Other innate immune proteins are likely to apply selective pressure on viruses, including hepaciviruses. This biological effect can be revealed by longitudinal analysis of the susceptibility of HCV isolates for the recognition of innate

proteins [183]. Until now, HCV strains resistant to defence collagens (ficolins and MBL) are yet to be described.

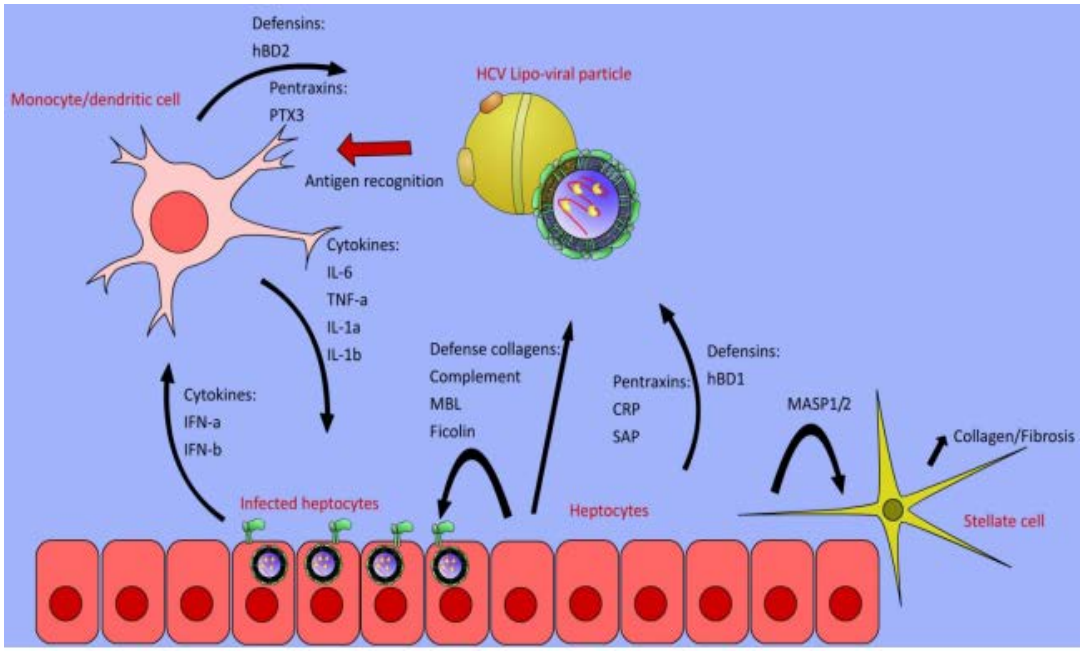

Figure 1-10 Overview of the production of soluble proteins involved in innate recognition of HCV antigens.

Antigen-presenting cells such as dendritic cells recognise viral antigens, resulting in the production of defensins and pentraxins, and pro-inflammatory cytokines TNF- α, IL-6, IL-α and IL-1β. Acute phase proteins in hepatocytes such as defensins and complement, which contribute to clearance of viruses and infected cells, are induced by cytokines. The hepatocytes are also a source of PRRs (defence collagens); MBL and ficolins. These proteins recruit MASPs which triggers the complement cascade [183].

1.11.7 Treatment of HCV infection and its impact

1.11.7.1 Standard treatment

Treatment of HCV is aimed at eradication of the infection to delay or prevent progression to cirrhosis, end-stage liver disease and hepatocellular carcinoma. This can be attained by achieving a sustained virological response (SVR), defined as the absence of detectable HCV-RNA in serum, 12 or 24 weeks after the completion of therapy [198-200]. Until 2013, the standard treatment for HCV was limited to pegylated interferon-alpha (PEG-IFN-α) and ribavirin (RBV) [201, 202]. The

guidelines for this treatment depended on viral genotype. The recommended dosage for all genotypes was the use of PEG-IFN-α2a at a weight-based dose of 1.5 µg/kg per week whereas patients infected with genotype 1, 4, and 6 received a daily dose of RBV for 15 mg/kg body weight. Patients infected with genotype 2 or 3 were treated with PEG-interferon and a flat dose of 800 mg of RBV daily, for 24 weeks [199, 201]. The SVR rates achieved with this regimen were relatively low, with less than 50% in HCV infected genotype 1 patients for 48 weeks and about 90% in genotype 2 and 3 patients for 24 weeks [203]. Significantly, it was associated with side effects, especially in genotype 1 infected patients [204, 205]. Therefore, there was a need to improve HCV treatment, which has led to the search for new antiviral drugs to improve HCV treatment efficacy.

1.11.7.2 Antiviral treatment

More recently, direct antiviral agents (DAAs) that target steps of the HCV life cycle have been introduced into the clinic. Four classes of DAAs have been defined by their mechanism of action and therapeutic targets; non-structural proteins (NS3/NS4A) protease inhibitors, NS5B nucleoside polymerase inhibitors, NS5B non-nucleoside polymerase inhibitors, and NS5A inhibitors [206]. They have activity against three viral proteins; the NS3/4A protease, NS5A and the NS5B RNA-dependent RNA polymerase (RdRp). The NS3/4A serine protease inhibitors (boceprevir and telaprevir) were the first DAAs approved to treat HCV genotype 1 infection, and improved the SVR rates up to 75% in combination with standard therapy (PEG-IFN-α and RBV) [202, 207, 208]. Although these drugs were effective, they were associated with side effects including itching and rashes. The second phase protease inhibitors such as Simeprevir were used in the treatment of chronic genotype 1 infection in combination with standard therapy but had an improved side effect profile with an SVR of 85-95% [209-211]. Sofosbuvir is a nucleoside analogue polymerase inhibitor, when used with the standard therapy resulted in greater than 90% SVR at 12 weeks in genotype 1 and 4 infected patients [212]. Dasabuvir is a non-nucleotide inhibitor of

HCV RdRp and its response rate is high (97-100%) in patients with genotype 1b [213-215]. Genotype 1a infected patients had lower responses but benefitted from the inclusion of RBV [216]. The NS5A inhibitors include Ledipasvir, Daclatasvir, Ombitasvir, Elbasvir and Velpatasvir. Daclatasvir has a pan-genotypic activity and its combination with sofosbuvir achieved high SVR rates in both naïve patients and those who had failed regimens from the first generation NS3/NS4A protease inhibitors [217]. Success has been achieved with Daclatasvir/Ledipasvir in combination with Sofosbuvir. These combinations treat HCV-infected patients infected with genotype 1, 2 and 3 with response rates greater than 90% [133]. However, new regimens have been recommended based on results from different phases of clinical trials in patients infected with genotype 1-6 [218]. Sofosbuvir/Velpatasvir and Glecaprevir/Pibrentasvir combinations have been recommended for all patients infected with genotype 1-6 [218]. Sofobusvir /Velpatasvir is a pan-genotypic regimen with > 95% of SVR across all genotypes [219, 220].

These DAAs are not limited to treatment of HCV but also include HIV/HCV co-infected patients. Targets have been set for the micro-elimination of HIV/HCV-coinfected patients using DAAs [221, 222]. With these developments, the treatment of chronic HCV infection with DAAs in developed nations is a step towards global eradication. However, the high cost makes them inaccessible for patients in need. Consequently, a prophylactic vaccine is needed. HCV entry could, therefore, serve as a target for inhibition as this involves few steps regarding virus-cell interaction.

1.11.8 Laboratory models for HCV entry systems

Several studies have shown consistent development of cell culture systems for the efficient production of HCV (Figure 1-11). As long as the HCV life cycle is concerned, pathogenesis and novel antiviral treatments were not satisfactory, as infection models were limited to infection of chimpanzees [223] and immunodeficient mice [224]. The development of retrovirus-based HCV pseudoparticles was important in the study of

virus entry into host cells. Expression vectors containing human immunodeficiency virus or murine leukaemia virus were used to produce HCV pseudotype particles bearing the E1/E2 glycoproteins. These particles contain a reporter gene which allows effective monitoring of infection into susceptible cells [225]. Another approach to viral entry into cells is the HCV cell culture (HCVcc) model, which can efficiently produce HCV infectious particles [143]. This system was derived from transfection of the human hepatocarcinoma cell line (Huh-7) with RNA of Japanese fulminant hepatitis (JFH-1) strain (genotype-2a isolate). The major cell culture systems used in the study of HCV are outlined below (Figure 1-11).

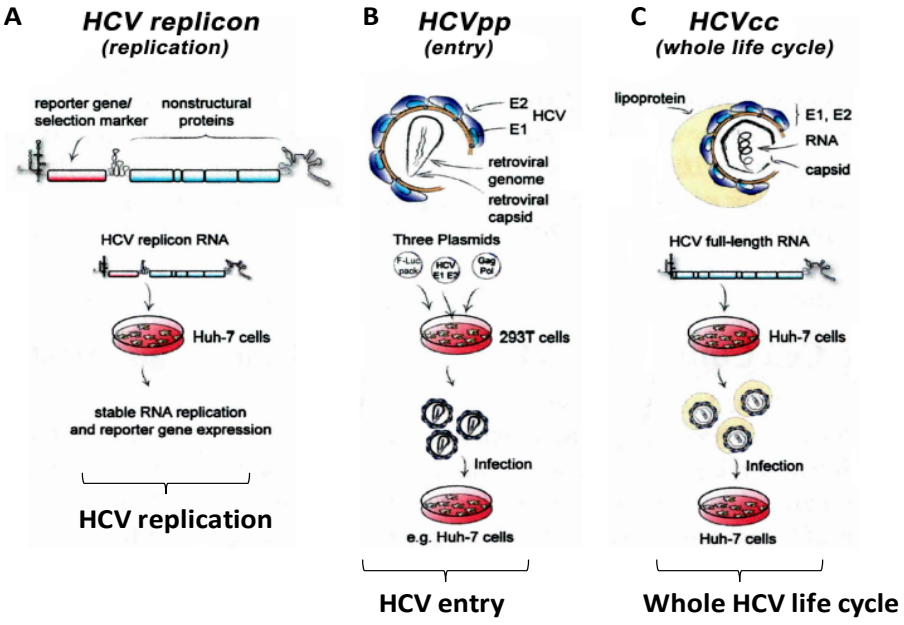

Figure 1-11: Outline of HCV cell culture systems to explore different steps of the viral life cycle

The various systems illustrated in the outline are used to investigate A) HCV replication B) HCV entry or C) the complete HCV lifecycle. This outline was adapted from the cell culture systems for HCV study [226].

1.12 **Non-primate/Equine Hepacivirus (Hepacivirus A)**

NPHV/Equine (also called hepacivirus A) was described for the first time in 2011 in domestic dogs [95, 96], and initially named canine hepacivirus (CHV) [95]. The virus was later discovered mainly in horses [97, 98, 227, 228]. Subsequently, the virus was renamed NPHV then equine hepacivirus (EqHV), and horses were presumed to be the natural host [95-97]. EqHV is an envelope, single-stranded, hepatotropic, positive-sense RNA virus that infects horses worldwide with a seroprevalence of 8 to 56% [228-230]. The genome of EqHV is like that of HCV, with a long open reading frame (ORF) of around 8,826 nucleotides encoding 2,942 amino acids. The EqHV ORF is predicted to produce structural proteins core, E1 and E2 and the non-structural proteins p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B [231, 232]. Although the ~384 nucleotides of the 5'-UTR resembles that of HCV, it has a single microRNA (miR-122) seed site and a longer stem-loop I [96]. It has also been confirmed that the predicted structure of the IRES is like that of HCV and can drive translation downstream of the ORF.

EqHV has similar pathogenesis to HCV during acute infection; however persistent infection occurs in <25% of horses based on prevalence and infection studies [229, 230, 233]. The virus displays distinct liver tropism with transmission by plasma transfusion and induction of hepatitis in horses [230]. The presence of viral RNA during hepatitis and persistent infection has been demonstrated [231, 234]. Coinfection with EqHV and equine pegivirus was recognised in association with liver disease in horses [235]. While HCV chronic infection occurs in the majority of cases, chronic EqHV infection has not been associated with clinical disease/progression as animals infected are able to clear the infection [98, 231, 234]. These findings were further supported by a recent study where a naturally occurring chronic infection with EqHV in a horse was reported [236].

This difference in infection outcomes and factors makes non-primate hepacivirus an attractive model system for HCV. EqHV is in its early years of research and several studies have established similarities between HCV and EqHV including molecular characteristics, mode of transmission, the course of infection, immunity and hepatotropism [98, 230, 237-239]. All these studies have contributed to the characterisation of hepaciviral infections, explaining the mechanisms of HCV and EqHV immunity. However, these investigations are limited by the lack of an *in vitro* system available for EqHV.

1.13 **Pseudotyped Viruses**

1.13.1 Overview

Pseudotyped viruses/pseudoparticles are chimeric virus particles composed of a core/backbone of one virus and at least one envelope protein or glycoprotein derived from another virus [240-242]. They encode a quantifiable reporter gene engineered into their genome. Following transduction of susceptible target cells, the envelope proteins interact with cell receptors permitting cellular entry, eventually resulting in the reporter gene's expression. Subsequently, the levels of the reporter gene expressed in the infected cells can be measured as a quantitative readout which directly correlates with the function of the envelope glycoprotein of the study virus [243]. Pseudotyped viruses cannot replicate continuously and are only capable of one round of replication because they lack the genetic components of replication. This enables them to be safely handled in low containment biosafety level 2 (BSL-2) laboratories.

Retroviruses, including lentiviruses (human immunodeficiency virus (HIV)) and gammaretroviruses (particularly murine leukemia virus (MLV)), as well as rhabdoviruses (vesicular stomatitis virus (VSV)) have been used as backbones for pseudotyped viruses. Retroviruses have proven advantageous owing to their ability to integrate foreign proteins which include host-derived proteins and envelope proteins of other viruses into their envelope membrane [244, 245]. After infection,

retroviruses deliver and integrate a deoxyribonucleic acid (DNA) copy of their RNA genome into the genome of the target cells during replication. This enables the reporter gene to be introduced into the retroviral genome alongside promoters, leading to the expression of an integrated reporter gene [240]. Although the HIV packaging system is the most widely used pseudotype packaging system, heterologous viral glycoproteins have also been pseudotyped onto VSV particles [246]. Cores from lentiviral HIV and gammaretroviruses (MLV) have been popular choices to produce HCV pseudotypes/pseudoparticles (HCVpp) [247].

1.13.2 Production of pseudotyped virus

Pseudotyped viruses can be produced by co-transfecting plasmids comprising the envelope gene, the retroviral *gag-pol* genes (the core) and a chosen reporter gene into a producer cell line such as human embryonic kidney 293T cells (HEK 293T) using a two or three-plasmid system. A two-plasmid system (Figure 1-12) comprises the core gene being incorporated into the same construct as the reporter gene and the envelope gene on a different plasmid while the three-plasmid system consists of each plasmid being transfected separately. The *gag-pol* genes are responsible for processing structural proteins (including matrix, capsid and nucleoprotein) and enzymatic proteins found within the core and the integration of the reporter gene into the host cell genome. Consequently, the packaging signal (Ψ), is excluded from the *gag-pol* construct to prohibit replication competence and any possible risk of pathogenic virus proliferation. In addition, particles could be produced with the *gag-pol* plasmid without supplying the envelope glycoprotein and/or the reporter gene as transduction control and this is often termed as ∆env (∆E). The reporter plasmid encodes the reporter protein, which contains a packaging signal upstream of the gene to facilitate the incorporation of the viral RNA into the host genome during pseudotype production. After the transcription and translation of the transfected genes by the cell machinery, an RNA dimer of the reporter gene is integrated into the pseudotype core

through the packaging signal. The pseudotyped core having integrated the two copies of the RNA reporter gene transits to the plasma membrane of the producer cell which contains the foreign viral protein bearing the heterologous glycoprotein expressed from the envelope gene plasmid. Pseudotyped particles are released into the extracellular medium after budding and encapsidation to form the viral envelope. The pseudotyped particles can be harvested from the cell supernatants and can be and titrated onto permissive target cells to measure the amount of functional particles using neutralisation assays.

A two-plasmid system lentiviral packaging construct (pNL4-3.Luc.R-E-) (Figure 1-12) was used in this study for the production of hepacivirus pseudoparticles (pp) and have been successfully used by different studies [247-251]. pNL4-3.Luc.R-E- is a replication-deficient proviral HIV-1 clone which has inhibitory frame shifts in the *env* and *vpr* genes [252] as well as the luciferase reporter gene cloned into the *nef* gene. The vector mimics the life cycle of HIV by using the Ψ to allow the encapsidation into pseudotypes and long terminal repeat (LTR) regions bearing the promoter which with the aid of the HIV transactivator (*tat*), enables the expression of the viral proteins after incorporation into the host genome.

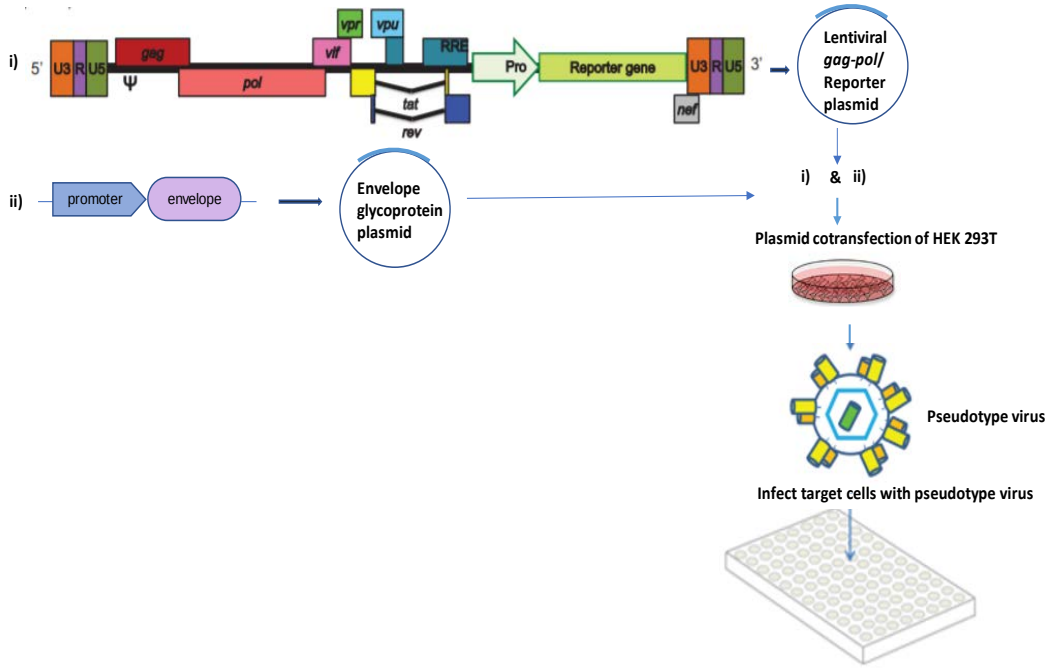

Figure 1-12 A two plasmid system for pseudotype virus production

A two-plasmid co-transfection method for pseudotype virus production with plasmid DNA expression vectors bearing i) pNL4-3.Luc.R-E- (*gag-pol*reporter gene). This has a frame shift in the *env* and *vpr* genes with luciferase reporter cloned into the *nef* gene. ii) the envelope glycoprotein from the virus of interest. The two plasmids are transfected into HEK 293 T cells (producer cells). The pseudotype virus is harvested and titrated unto susceptible target cells. The pNL4.3 reporter gene figure was adapted from a study on pseudotype based-neutralisation assays [253].

1.13.3 Application of pseudotyped viruses

Pseudoviruses are widely used at lower containment laboratories (regardless of the virus envelope protein) and have been used for the investigation of highly pathogenic viruses requiring higher containment. They are suitable for a wide range of applications and provide a safe, flexible platform since they cannot replicate apart from the reporter gene maintained as the genome. Pseudotyped viruses are used for research and therapeutic agents screening, measurement of antibodies, vaccines and gene transfer and these have all been reviewed by different studies [240, 243, 254, 255]. Retroviral pseudotypes are used in understanding the neutralisation of virus entry. Retroviral pseudotype virus bearing HCV E1 and E2 protein (HCVpp) has been

widely used to investigate the neutralisation of HCV entry. The pseudotype neutralisation assay was established to test the neutralising potency of human sera [256-258] and monoclonal antibodies [259-262] against different strains of HCV. More studies using the HCVpp showed that there were other factors such as PRRs (ficolins) contained in the serum other than antibodies that have the potential to enhance or neutralise HCV entry [89, 149, 193] and protect against antibody neutralisation [263]. Successful transduction of the target cell by the pseudotype virus leads to the expression of the reporter gene. However, in the presence of a neutralising antibody (or glycoprotein-binding ficolin) the interaction of the surface glycoprotein on the pseudotyped virus to the cell surface is inhibited. Hence, neutralisation can be measured as the decrease in reporter gene signal relative to the pseudotyped virus infection without neutralising antibody/ficolin. Pseudotyped virus neutralisation assays have been developed for other viruses including influenza and Ebola [15, 264] to test the neutralising potency of human ficolins.

The robustness and flexibility of retroviral pseudotypes means that they can be used to identify receptor usage and tropism of newly discovered pathogens like EqHV. In addition, the availability of retroviral pseudotypes as an entry model may contribute to the characterisation between entry inhibitors (such as ficolins) and viral envelope proteins.

1.14 **Aims and Hypothesis**

The aim of this project was to assess the direct anti-viral properties of ficolins expressed by different mammalian species. The open reading frames of cDNA encoding mouse ficolins (FCNA and FCNB) and NHP ficolins-2 (gorilla and colobus monkey) were cloned, expressed and purified. They were characterised to determine their potency for binding and neutralizing virus particles. The ability of the recombinant proteins to bind to hepacivirus glycoproteins E1/E2 was investigated. This study also involved the development of a pseudotype assay to investigate hepacivirus entry into host cells.

It is hypothesised that ficolins from non-human species may have varying ability to neutralise hepaciviruses better than human ficolin-2. Some host immune proteins have been shown to limit infection of different viruses, applying immune selective pressure and selecting for variants that must demonstrate some resistance to recognition in order to allow persistent infection. Therefore, viruses that have evolved in the absence of specific host proteins such as ficolins might be recognised and neutralised better than those viruses persistently exposed during replication. Hence, proteins possessing different recognition and binding domains of the different species of ficolins may have potential as novel inhibitors of virus entry.

2 Characterisation of the Evolution of Ficolin Genes in Mammalian Species

2.1 Aims

In this chapter, the aim was to investigate the current phylogenetic and inter-individual molecular evolution of the ficolin genes from selected mammalian species. Mammalian species were selected to represent ficolin-2 (NHP) and mouse ficolins; A and B (Figure 2-1). Since only limited knowledge of the other mammalian species of ficolins exists, it was important to sequence the coding region of non-human primate ficolin-2 and mouse ficolin genes. The conservation and variability of the sequences were analysed to determine their effects on the protein structure and function. This study aimed to create His-tagged expression constructs, perform phylogenetic analysis and compare the ligand binding abilities between ficolin species. Results from these experiments were compared to the well-studied human ficolin-2.

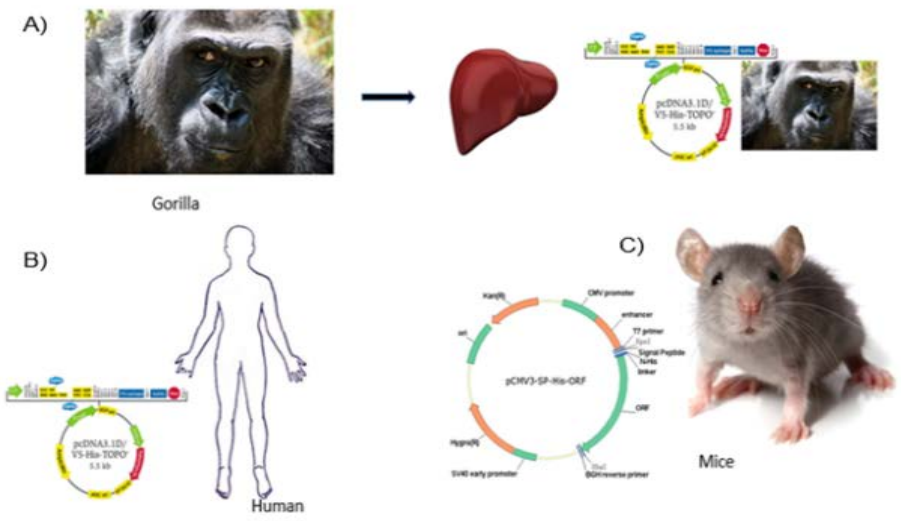

Figure 2-1 Overview of ficolin constructs used for this study

The schematic shows the different mammalian ficolin constructs used in this study. A) Non-human primate ficolin construct produced by extracting RNA from the liver which was used to synthesise cDNA. The cDNA was used as a template for the amplification of non-human primate ficolin. B) A human ficolin-2 which had been successfully cloned into a human expression vector (pCDNA 3.1) previously in our research group. C) Mouse ficolin A and B constructs which were obtained commercially.

2.2 Materials and Methods

2.2.1 Materials

2.2.1.1 Source of Samples

Seventy-six liver samples of NHP were collected from Twycross Zoo after local ethical approval was obtained. Three NHP species were selected for RNA analysis; marmoset (*Callithrix jacchus*), gorilla (*Gorilla gorilla*) and colobus monkey (*Colobus angolensis*) (Table 2-1). Cloned N-His tagged mouse ficolins A (FCNA) and B (FCNB) in an expression vector pCMV3-SP-N-His were obtained from Sino Biological Inc. Finally, Dr Mason; a former PhD student at the Virus Research Group, donated 2 DNA expression constructs encoding human ficolin-2 with either C-terminal (E4) or N-terminal (D4) His-tag labels. The N-terminal His-tag human ficolin-2 expression construct was used as a positive control in this study having been successfully used for the expression of human-ficolin 2 protein in our research group.

Table 2-1: Details of primate samples used in this study

VRD No	Date	Identifier	Sample	Species
H06 336 0621 01	23/08/2006	06-0192	Post-mortem-tissue	Geoffrey's marmoset
H06 336 0625 01 x2	23/08/2006	05-0552	Liver	Black and white Colobus
H08 122 0608-08 06x2	26/02/2007	07-0325	Liver	WL Gorilla

2.2.1.2 Primers

Primers were designed in-house for the successful amplification of DNA by using the Primer3, OligoAnalyzer 3.1 and Molecular Evolutionary Genetics Alignment (MEGA) version 6 [265] tools and then synthesized by Eurofins Genomics.

Table 2-2: Primers used in the amplification of NHP ficolin and control genes

Primer Designation	Primer Sequence (5'- 3')
NHP_GAPDHF	GAAGGTGAAGGTCGGAGTCA
NHP_GAPDHR	CATGAGTCCCTTCCACGATACC
GC2FCN2_F	ATGGAGCTGGACAGAGCTG
GC2FCN2_R	CTAGGCAGGTCGCACCTTCAT
MAR2FCN2_F	ATGGGACCCGGTCTCCTG
MAR2FCN2_R	CTAGGCGGGCCGCACCTTC

Table 2-3: Screening primers used in this study

Primer Designation	Primer sequence
M13F	TGTAAAACGACGGCCAGT
M13R	CAGGAAACAGCTATGACC
T7F	TAATACGACTCACTATAGGG
BGHR	TAGAAGGCACAGTCGAGG
NHPSPHIS- F	CACCCCTGCTGCTCACTTTC
NHPSPHIS- R	TCCATGCACAGAGCACAGT

Table 2-4: Primers used for the creation of His-tagged recombinant NHP

Primer Designation	Primer Sequence (5'- 3')
FCN2SPCG1_F (P1)	CACCATGGAGCTGGACAGAGCTG
FCN2HisSPCG2_R (P2)	ATGGTGATGGTGATGATGAGCCCAGGCCATGCCCAGG
FCN2HisSPC3_F (P3)	CATCATCACCATCACCATGTCCAGGCGGCAGACACC
FCN2HisSPG3_F (P3)	CATCATCACCATCACCATCTCCAGGCGGCAGACACC
FCN2StrpSPCG4_R (P4)	CTATTTTTCGAACTGCGGGTGGCTCCAGCTGCCCCCGGCAGGTCGCACCTTCAT

2.2.1.3 Plasmids and Kits

pGEM®-T Easy vector (Promega) was used for cloning and sequencing of PCR products. The pcDNA3.1 Directional TOPO Expression kit (Invitrogen) was used for protein expression in tissue culture assays. Primers were designed to produce a blunt-end PCR product of NHP ficolin cDNA with the introduction of CACC in front of the start codon. The primers (P1-P4) (Table 2-4) were used together in a PCR and the pGEM®-T Easy vector NHP ficolin as a template. The PCR-amplified product was

TOPO-cloned into pcDNA3.1D/V5-HisTOPO vector (Invitrogen), yielding the pcDNA3.1D/ V5-His-NHP-ficolin expression construct.

2.2.2 Cloning of the NHP ficolin-2 gene

2.2.2.1 RNA extraction of NHP samples

RNA was recovered from approximately 40 mg of frozen liver using the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich). The liver from each NHP was homogenised in 500 µL lysis solution using a hybrid ribolyser homogenizer at speed 6 for 40 secs preceding extraction. The lysis reaction is very critical to avoid RNA degradation; hence the reaction was carried out as quickly as possible. The homogenized lysates were filtered through a filtration column (CP9346) and centrifuged at 2000 x g for 2 minutes to remove debris and shears of DNA. Five hundred microlitres of 70% ethanol was added to the filtered lysate to allow binding to the GenElute™ column. A three-step wash was carried out and RNA was then eluted with a 50 µL elution buffer. The final concentration and quality of RNA were determined by spectrophotometric analysis using a Nanodrop; ND-1000 model. RNA samples were used for cDNA synthesis immediately or stored at -80^{o}C.

2.2.2.2 Complementary DNA (cDNA) synthesis

NHP ficolin cDNA was synthesised using a commercially available RevertAid™ Reverse Transcriptase kit (Thermo Scientific) to generate a first strand cDNA for a two-step Reverse Transcription Polymerase Chain Reaction (RT-PCR). In the first step, RNA (template), 2 µL of Oligo (dT) (primer) and 8.5 µL of water mix was incubated at 65^{o}C for 5 minutes. The mix was placed on ice afterwards. This step removes secondary structures that can obstruct cDNA synthesis. In the second step, denatured RNA was combined with 7.5 µL extension master mix, consisting of 4.5 µL 5X reaction buffer, 2 µL dNTP and 1 µL of RT and incubated for 60 minutes at 42^{o}C.

The reaction was terminated at 70ºC for 10 minutes. The final product (cDNA) was used immediately or stored at -20ºC.

Primers were designed (NHP_GAPDHF and NHP_GAPDHR - Table 2-2) for the amplification of NHP cDNA template to confirm the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the housekeeping control gene. A 520 bp amplicon size was expected from a reaction of 12.5 µL with cycling conditions; 95ºC for 15 minutes, 95ºC for 20 secs, 54.4ºC for 30 secs for 50 cycles, 72ºC for 30 secs and 72ºC for 30 secs.

2.2.2.3 PCR amplification of NHP FCN2

An alignment of NHP and human ficolin-2 mRNA sequences was made from sequences publicly available in GenBank using the Clustal W algorithm as implemented in Molecular Evolutionary Genetics Analysis (MEGA) version 7. Primer sets were designed for the amplification of NHP ficolin-2. Marmoset was amplified with a primer set (MAR2FCN2_F and MAR2FCN2_R) with an expected amplicon size of 1,272 bp while colobus and gorilla used the same primer set (GC2FCN2_F and GC2FCN2_R) with an expected amplicon size of 828 bp (Table 2-2). The GC2FCN2 primer set was used for the amplification of the positive control (human ficolin -2) with an expected amplicon size of 942 bp. Reaction volumes of 12.5 µL were prepared to contain 10X PCR buffer 1.25 µL, forward and reverse primer 0.5 µL each, 0.5 µL HotStarTaq DNA polymerase (Qiagen), 0.5 µL deoxynucleotide triphosphates (100 mM dNTPs) with human ficolin-2 construct donated by Dr Chis Mason used as a positive control. A negative control was set up by substituting the cDNA template with water. Amplification was carried out for 50 cycles at 95ºC for 15 minutes, 95ºC for 20 secs, 53.8ºC (colobus and gorilla), 55.5ºC (marmoset) for 30 secs, and 72ºC for 30 secs. This PCR reaction was set up to amplify the coding region of NHP FCN2 and human ficolin-2 encoding sequence.

The above amplification of NHP FCN2 gene was optimised using a gradient PCR to find the best suitable temperature for annealing the primers. The same reaction volume was used as above, however, 8 strips of tubes were used for the reaction mix of each ficolin. The gradient PCR was carried out using a temperature range of 54 to 60^{o}C.

2.2.2.4 PCR clean up, agarose gel electrophoresis and DNA sequencing

PCR products were cleaned up using the NucleoSpin® Gel and PCR clean-up kit, according to the manufacturer's instructions. PCR products were analysed by gel electrophoresis, separated on 2% agarose gel at 90 V for 30 minutes. Amplicons expected at 828 bp product length were sent for sequencing to the Source Bioscience, Nottingham, UK using GC2FCN2 forward and reverse primers (Table 2-2) for colobus and gorilla. Sequencing data were analysed using Finch TV, after which they were searched on the National Centre for Biotechnology Information (NCBI) database to identify consensus sequences.

2.2.2.5 PCR subcloning into pGEMT easy vector system

The successfully amplified insert from section 2.2.2.3 above was subcloned into a pGEM®-T Easy Vector. The reaction was set up by mixing 1 µL of the amplified product with 1 µL pGEM®-T Easy Vector (50 ng/µl), 5 µL of 2X Rapid Ligation Buffer and 1 µL of T4 ligase. Two microlitres of deionized water was added to make up to a final volume of 10 µL. The reaction was incubated at 22^{o}C for 1 h. This was then transformed using competent cells as described below, section 2.2.2.6. Subsequently colony PCR was completed to identify transformants using M13F/M13R or gene specific (GC2FCN2) primers (Table 2-2 & Table 2-3) and analysed on agarose gel. Transformants containing the plasmid of interest were inoculated into 7 mL LB broth (with ampicillin) and incubated overnight at 37^{o}C and 220 rpm with shaking. Plasmids were purified using GenElute^{TM} plasmid kit and measured by nanodrop spectrophotometer.

2.2.2.6 Transformation of competent cells

Competent cells were thawed on ice and 25 µL were added to 2 µL of ligation mixture in a 1.5 mL microcentrifuge tube. This was mixed gently by pipetting and the resulting mixture was incubated on ice for 20 minutes. This was followed by a 'heat shock' at 42^{o}C for 30 secs and further incubation on ice for 2 minutes. Two-hundred and fifty microliters of super optimal broth with catabolite repression (SOC) medium was added to the transformed cells and incubated with shaking for 1 h at 37^{o}C and 220 rpm. This was followed by inoculation on a prewarmed Luria Bertani (LB) agar medium (containing 100 µg.mL^{-1} ampicillin) and incubated at 37^{o}C overnight.

2.2.2.7 Analysis of transformants

A directional PCR was used to analyse positive transformants that grew on the ampicillin-containing LB agar plate. PCR reaction was prepared with two set of primers; vector specific primers M13F and M13R or the ficolin forward and M13R primers. Inserts were amplified with HotStarTag using the following thermal cycling parameters; 95^{o}C for 15 minutes, 94^{o}C for 45s, 50^{o}C for 45s, 72^{o}C for 3 minutes and 72^{o}C for 7 minutes**.** Amplified products were analysed on a 2% ethidium bromide-stained agarose gel and sequenced afterwards.

2.2.2.8 Plasmid DNA mini and midi preparation

Colonies containing the correct orientation of the ficolin insert were inoculated into 5 mL or 50 mL LB culture containing 100 µg.mL^{-1} ampicillin and shaking at 225 rpm overnight at 37^{o}C. Cultures were used to extract plasmids and purified using GenElute^{TM} miniprep or midiprep, following all manufacturer's instructions duly. It was also used to prepare glycerol stock for long time storage at -70^{o}C.

2.2.2.9 Creation of a double-tagged NHP ficolin-2 construct

A recombinant N-terminal His-tagged and C-terminal Strep-tagged colobus and gorilla FCN2 constructs were created by inserting a nucleotide sequence encoding a 6X His-tag (CATCATCACCATCACCAT) downstream of the signal peptide cleavage site to avoid post-translational processing effects. The cleavage site was detected by using SignalP prediction tool. Appendix 1 (section 7.1) shows a schematic representation of the constructs. The constructs were double tagged to enable purification of the protein from either the N or C-terminal.

2.2.2.9.1 Fusion PCR

The tagged recombinant NHP FCN2 was produced by fusion PCR (Figure 2-2). This PCR involved two rounds; in the first round, fragment A was created using NHP ficolin forward (P1) and His-tag antisense (P2) primers and fragment B, using His-tag NHP ficolin sense (P3) and Strep-tag NHP ficolin antisense (P4) primers (Table 2-4). A 12.5 µL reaction volume was prepared to contain 10X PCR buffer 1.25 µL, sense primer (P1/P2) and antisense primer (P3 and P4) 0.5 µL each, 0.5 µL HotStarTaq DNA polymerase (Qiagen) and 0.5 µL dNTPs with human ficolin-2 plasmid construct used as a positive control. The same materials where the template was substituted with water was used as a negative control. Amplification was optimised by using a gradient PCR carried out for 35 cycles at 95^{o}C for 15 minutes, 95^{o}C for 20 secs, 60 to 72^{o}C (colobus and gorilla); for 30 secs (where column 1-8 where; 60.9^{o}C, 62.0^{o}C, 63.4^{o}C, 65.4^{o}C, 65.2^{o}C, 67.2^{o}C, 68.9^{o}C, and 70.2^{o}C), and 72^{o}C for 3 minutes. Colobus was repeated using the same conditions as stated above but for 25 cycles, at an annealing temperature of 58^{o}C and an extension of 2 minutes. PCR products were confirmed by agarose gel electrophoresis.

In the second-round PCR, the amplicons from fragments A and B were used as primers and templates to produce the N-terminal His-tagged NHP ficolin. The same conditions as in the first-round amplification were used. The recombinant NHP ficolin

was confirmed by agarose gel electrophoresis. After optimisation, the annealing temperature for the first and second round fusion PCR were confirmed at 62ºC and 66ºC, respectively. Hence fusion PCR of NHP cycling conditions for both His-tag and Strep-tag inserts were; first-round PCR for 35 cycles at 95ºC for 15 minutes, 95ºC for 30 secs, 62ºC (colobus and gorilla) for 30 secs, 72ºC for 30 secs and 72ºC for 3 minutes. Second-round PCR for 25 cycles at 95ºC for 15 minutes, 95ºC for 20 secs, 66ºC (colobus and gorilla) for 20 secs, 72ºC for 30 secs and 72ºC for 2 minutes.

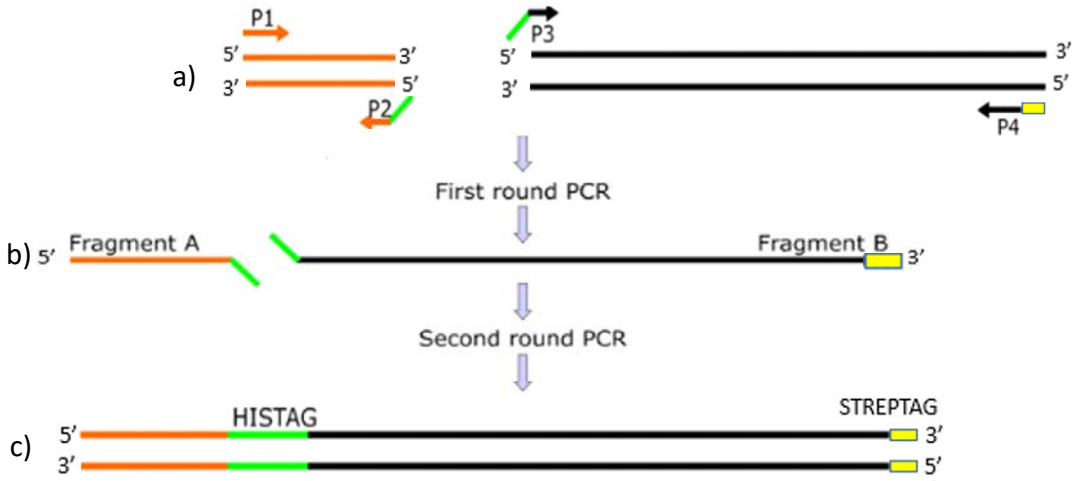

Figure 2-2: Schematic outline of fusion PCR used for the generation of NHP His-tagged ficolin

a) First round PCR where two sets of primers were used: P1- (NHP forward) and P2- His-tag antisense which yielded fragment A (including the TOPO directional sequence, signal peptide and part of the His-tag). P3 (using His-tag NHP ficolin sense) and P4 (Strep-tag NHP ficolin antisense) to create fragment B b) Fragments A and B were combined in the second round PCR to produce the coding region of NHP ficolin (c) including both His and Strep-tags.

2.2.2.10 Creation of blunt-ended DNA product

The inserts from the fusion PCR were blunted to eliminate 3' and 5' overhangs for the promotion of blunt end ligation into a blunt-ended vector. This was achieved by the Q5® Hot Start High-Fidelity 2X Master Mix (New England Biolabs (#04945)) which is a hot start DNA polymerase with 3′→ 5′ exonuclease activity for proofreading. Production of the blunt end DNA product was carried out in a two-step PCR with 5 µL of the PCR products and 5 µL of the 2 X Q5 master mix. The mixture was incubated

at 98ºC for 30 secs and 72ºC for 5 minutes. The resulting product generated is blunt ended and ready for cloning.

2.2.2.11 Generation of expression constructs

The resultant blunt-ended PCR product was digested with DpnI to avoid background plasmid used as a template in the PCR moving along with PCR product. This blunt-ended PCR product was cloned into pcDNA3.1D/V5-His TOPO vector (Invitrogen life technologies) according to the manufacturer's protocol yielding the pcDNA3.1D/V5-His-NHP ficolin expression construct. The pcDNA3.1D/V5-His-NHP ficolin expression constructs were transformed and propagated into a competent *E. coli* and incubated at 37ºC overnight. Positive clones were analysed on ampicillin-containing LB-agar plates (100 $\mu g.mL^{-1}$). The recombinant NHP-ficolin insert was verified by directional PCR, using T7 forward and BGH reverse primers or NHP specific screening primers (NHPSPHIS- F and NHPSPHIS- R) shown in Table 2-3. Purification of plasmids was carried out using a Midi-Prep kit (Sigma) according to manufacturer's instructions.

2.2.3 Sequence and phylogenetic analysis of ficolins from different mammalian species

Mammalian ficolin nucleotide sequences were downloaded from the NCBI nucleotide database. Alignments of derived protein sequences and generation of identity/similarity matrices were performed with the ClustalW alignment tool of the MEGA 7 software.

2.2.3.1 Phylogenetic tree construction

Different predicted sequences of ficolins from different mammalian species were obtained from NCBI and aligned by ClustalW [266]. Most important were human, gorilla and mouse ficolins with accession numbers; NM-004108.3, XM-0040448836.2, XM- 006497673.3 (FCNA) and NM-010190.1, respectively. Using MEGA 7 tool [267],

a phylogenetic tree was constructed using the maximum likelihood method based on the Tamura-Nei model [268]. Bootstrap analysis was inferred from 1000 replicates to show the reliability of the branching patterns [269]. All positions containing gaps and missing data were eliminated.

2.2.3.2 Sequence alignment and analysis

An amino acid sequence identity matrix from different mammalian species of ficolin was computed using a sequence analysis tool-BioEdit version 7.5.0.3 [270]. The mammalian species of interest representing ficolin 1, 2, A and B had percentages above 50% when compared to human ficolin-2 and these were further analysed. The multiple alignments of the selected ficolins were performed by Clustal W omega and CLUSTAL W [271] according to their amino acid translation using MEGA [267]**.**

To investigate how the amino acid sequence has evolved, a pair-wise comparison between the nucleotide sequences was carried out. The Nei and Gojobori model [272] in MEGA 7 was used to calculate the nucleotide sequence distance in a pair-wise comparison. The distance was determined by the number of synonymous nucleotide substitutions per synonymous site (dS) and the number of non-synonymous substitutions per non-synonymous site (dN). Subsequently, the dN/dS ratio was calculated manually.

2.2.3.3 Conservation Analysis

A conservation analysis was carried out following the procedure described in a calculation of evolutionary conservation [273] using the consurf server [274]. The known protein structure of human ficolin-2 with protein data bank (PDB) code; 2j3g [12] was uploaded into the server and it automatically extracted the sequence and aligned it with the multiple sequence alignment uploaded. The multiple sequence alignment contained the selected mammalian ficolins generated in section 2.2.3.2 above. The degree of conservation was calculated, and the scores were mapped unto

a 3D structure. The structure was visualised and annotated using UCSF Chimera program [275].

2.3 **Results**

2.3.1 PCR amplification and cloning of NHP ficolin gene

2.3.1.1 GAPDH mRNA expression (Housekeeping gene)

To confirm the purity and integrity of the extracted RNA, specific primers (NHP_GAPDHF and NHP_GAPDHR) were used to determine the expression of GAPDH using the NHP cDNA as a template. GAPDH is a housekeeping gene often used as a reference gene as it is expected to be expressed consistently in most tissue samples [276]. The agarose gel electrophoresis showed bands at 520 bp specific for each NHP (Figure 2-3). This result suggests the successful amplification of GAPDH.

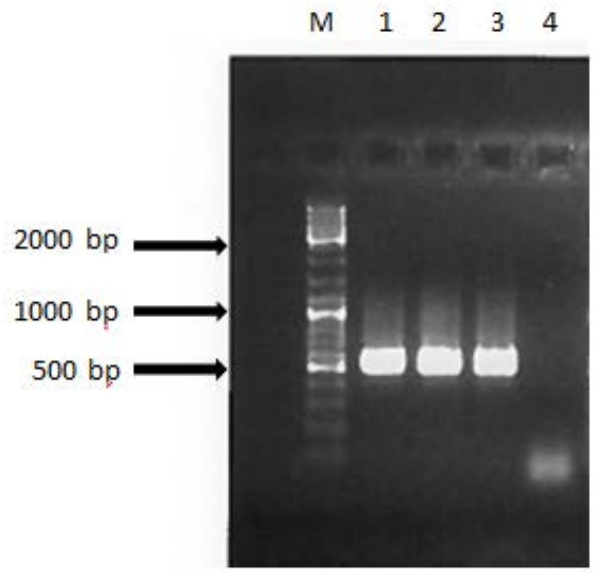

Figure 2-3: Agarose gel of GAPDH mRNA expression

A 100 base pair DNA size marker (M) is loaded in the first lane. Gel electrophoresis confirmed the expression of GAPDH at 520 bp using specific NHP GAPDH forward and reverse primers for lane 1, 2 and 3 which contain gorilla, marmoset and colobus cDNA respectively. Lane 4 is a negative control without a template and should, therefore, contain no bands.

2.3.1.2 Detection of NHP FCN2 gene

Gene-specific primers were designed to amplify the entire coding region of NHP FCN2 from 5' to 3' end from an NHP liver cDNA. The PCR primers; GC2FCN2_F and GCFCN2_R (colobus and gorilla) and MAR2FCN2_F and MAR2FCN2_R (marmoset) define an 828 bp and 1,272 bp PCR product, respectively. As shown in Figure 2-4, lane 7-9 represent different dilutions (10^{-6} to 10^{-8}) of the positive control (human ficolin-2 construct) which was detected by the GC2FCN2 primer set. Although specific bands were detected at 828 bp for gorilla and colobus (lane 3 and 4), there were primer dimers below 100 bp and non-specific bands at 300 bp and 500 bp (Figure 2-4). Speculation was made about the 500 bp, which might be a spliced variant of the gorilla FCN2. As there was no evidence to prove the spliced variant, this was not explored further.

In order to find the best annealing temperature possible, a gradient PCR of temperature range 54-60^{o}C (Figure 2-5) was used to optimise the reaction, ensuring accuracy and reproducibility for trusted results. The same materials as used in the conventional PCR were used, whilst human ficolin-2 construct was used as a positive control, a negative control with no template was also included. Non-specific bands were still present even at higher temperatures. Hence, the inserts from gorilla and colobus (lane 6 and 7 (Figure 2-5 i) & ii) were isolated by cutting the bands out of the agarose gel and purifying the DNA using NucleoSpin Gel and PCR clean up kit.

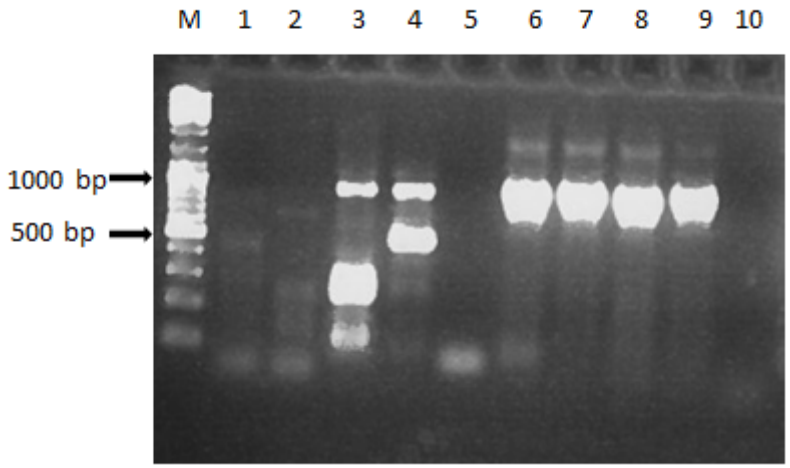

Figure 2-4: Detection of NHP ficolin-2 by PCR

PCR amplification of the full-length coding region of NHP ficolin-2 cDNA using GC2FCN2 and MAR2FCN2 forward and reverse primers. A 100 base pair DNA size marker (M) is loaded in the first lane. Lane 1 = non-specific bands for marmoset while lane 2 = negative control with no template. Lane 3 = colobus FCN2; 828bp, lane 4 = gorilla FCN2; 828bp with primer dimers below 100 bp and non- specific bands at 300 bp and 500 bp as seen in lane 3 and 4, respectively. Lane 5 = negative control with no template. The positive human ficolin-2 plasmid control with the same primer set is confirmed with neat (lane 6) and at a serial dilution of 10^{-6} to 10^{-8} from lane 7 to 9. Lane 10 = negative control with no template.

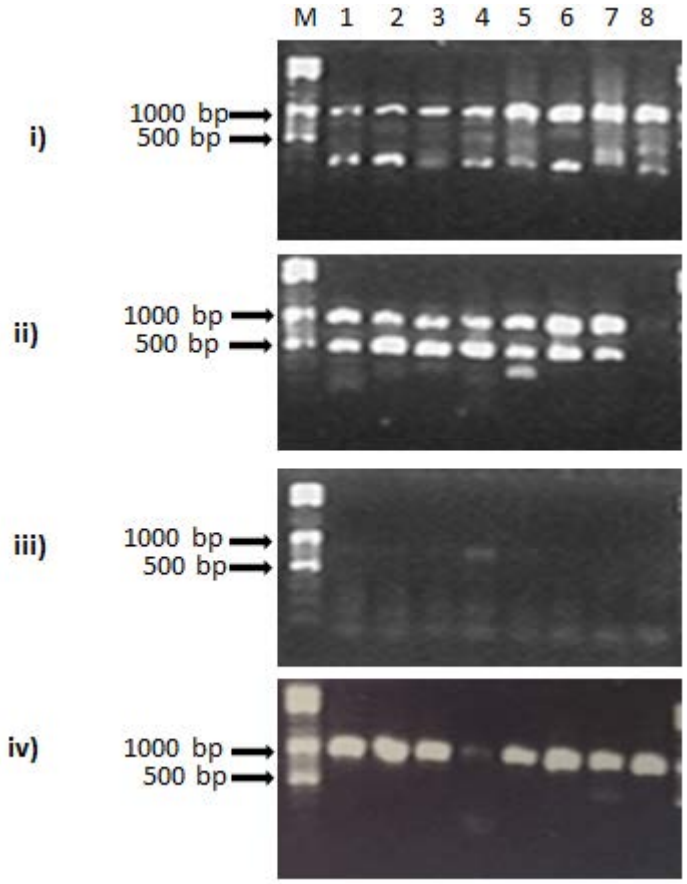

Figure 2-5: Gradient PCR showing the optimisation of ficolin-2 gene amplification from NHP species

Gradient PCR amplification of the full-length NHP ficolins cDNA; i) colobus ii) gorilla iii) marmoset iv) human ficolin-2 (positive plasmid control) using GC2FCN2 and MAR2FCN2 forward and reverse primers at a temperature range of 54–60^{o}C. A 100 base pair DNA size marker (M) is loaded in the first lane. Lanes 1 to 8 have a calculated gradient temperature of 54.0^{o}C, 55.0^{o}C, 55.7^{o}C, 56.6^{o}C, 57.60^{o}C, 58.5^{o}C, 59.1^{o}C and 59.9^{o}C respectively. Colobus (i) and gorilla (ii) ficolin-2 were detected at 828 bp but with the presence of non-specific bands. In marmoset (iii), light bands at about 800 bp below the expected band size (1,272 bp) at lane 4 were observed. A positive control (iv) was set up to ensure successful amplification. Also, a negative control (data not shown) with no DNA template contained no bands.

2.3.1.3 Successful cloning into PGEMT

Having successfully amplified the full-length NHP FCN2 cDNA, amplicons were subcloned into a PGEM-T plasmid vector and used to transform competent *E. coli* cells. Following an overnight culture, 8 colonies were selected and screened by PCR using the vector-specific primers; M13 forward and M13 reverse to confirm the presence of insert. No positive transformants of the NHP ficolin ligated into pGEMT were observed (Figure 2-6). This non-specific amplification is possibly due to the

presence of PCR inhibitors or possibly the non- specific binding of primers to partially matching regions.

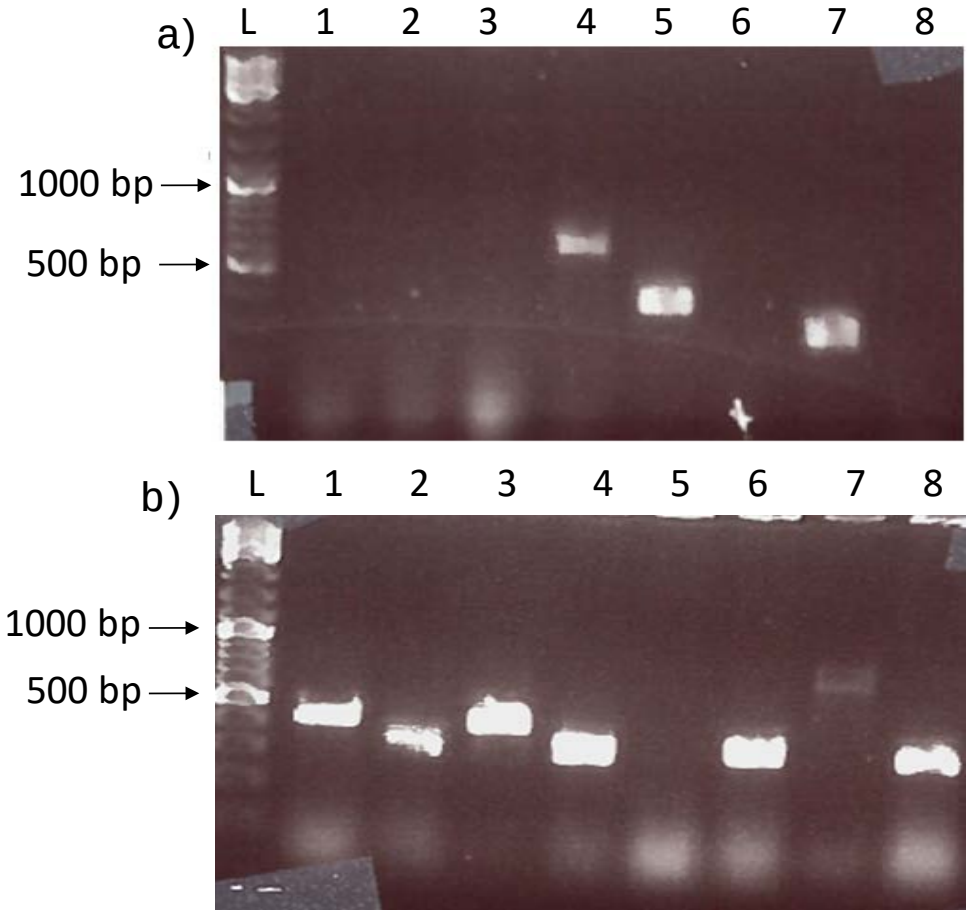

Figure 2-6: Screening of colonies for positive transformants

Eight colonies were screened by PCR using M13 forward and M13 reverse primers for positive transformants. The primers used were vector specific and are found in particular position in the vector to give 263 bp. When added to the inset size of 828 bp, the expected size was 1091 kb. No positive transformants were observed in both colobus (a) and gorilla (b) containing the ficolin insert.

A directional PCR was carried out with insert specific primers; GC2FCN2 forward and GC2FCN2 reverse primers. Positive transformants confirmed the presence of insert (Figure 2-7). Negative controls containing no DNA template showed no bands (data not shown). Sequencing revealed that the correct inserts were present in these plasmid clones.

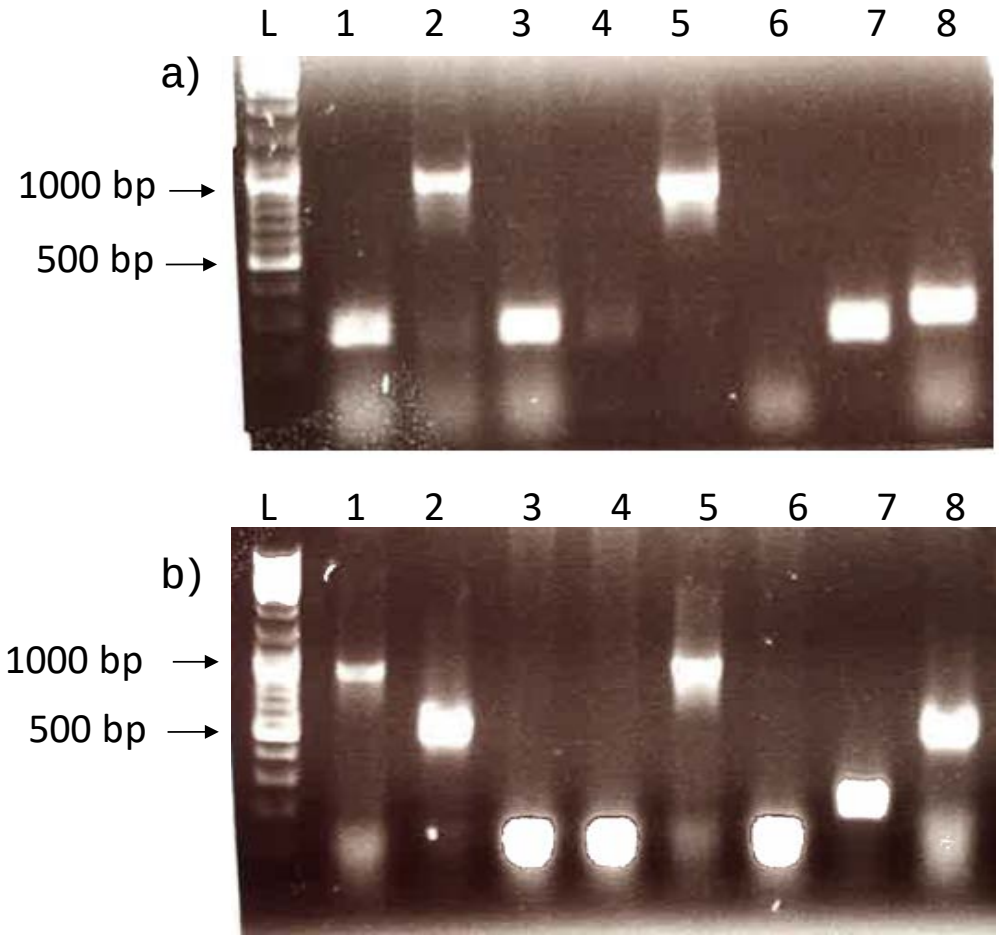

Figure 2-7: Directional PCR with specific insert primers for positive transformants

Colonies from Figure 2-6 were re-screened using insert specific primers; GC2FCN2 forward and GCFCN2 reverse primers. A positive amplicon was expected above insert size (828 bp). Lane 2 and 5 (a) confirmed colobus ficolin insert while lane 1 and 5 (b) confirmed the gorilla ficolin insert in the correct orientation of the insert.

2.3.2 Functional characterisation of NHP FCN2

To functionally characterise NHP FCN2, the full-length cDNA cloned into PGEM-T was subcloned into an expression vector; pcDNA3.1D/V5-His-TOPO. This was achieved by introducing His-tag and Strep-tag sequences using the pGEMT-NHP plasmid as a template using fusion PCR. The expression constructs were subsequently analysed in the next chapter. The cloning strategies are detailed below.

2.3.2.1 Creation of His-tagged and Strep-tagged Gorilla and Colobus Constructs

The signal peptide cleavage site was predicted using the Signal P 4.0 server prediction tool [277]. The cleavage site location was determined to be between amino acid positions 27 and 28. Thus, the insertion of the N-terminal His-tag was designed immediately downstream of the cleavage site. N-terminal His-tagged and C- terminal Strep-tag gorilla and colobus ficolin constructs were produced by fusion PCR (Figure 2-2)**.** Amplicons for both colobus (Figure 2-8 (i & ii)) and gorilla (Figure 2-9 (i & ii)) in the first-round PCR were independently combined in the second-round PCR to produce His and Strep-tagged colobus (Figure 2-8 (iii)) or gorilla (Figure 2-9 (iii)) ficolins. Having confirmed the annealing temperature range with the gradient PCR, the inserts were successfully fused at an annealing temperature of 62^{o}C and 66^{o}C for first and second PCR rounds, respectively (Figure 2-10). Both colobus and gorilla inserts were confirmed at the expected sizes.

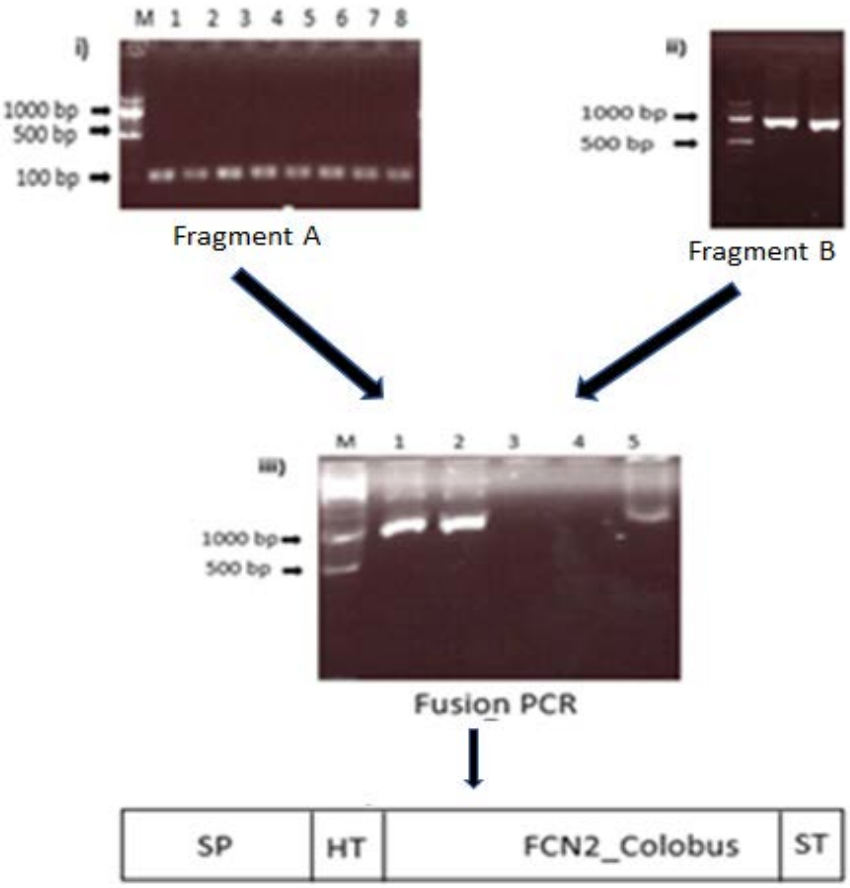

Figure 2-8: Colobus ficolin fusion PCR products:

Fragment A (lane 1-8) (i) using colobus ficolin forward and His-tag antisense primers by gradient PCR. Fragment B (lane 1-8) ii) using His-tag colobus ficolin sense and Strep-tag colobus antisense primers. The expected sizes of colobus ficolin were detected at 97 bp (Fragment A) and 918 bp (Fragment B). The second-round PCR was a combination of fragment A and B (iii) where lane 1 was with the Strep-tag colobus ficolin antisense primer and lane 2 was without the Strep-tag at an annealing temperature of 58ºC. The DNA size marker (M) is shown in base pairs (bp). Lane 1-8 represents annealing temperatures of 60.9ºC, 62.0ºC, 63.4ºC, 65.4ºC, 65.2ºC, 67.2ºC, 68.9ºC, and 70.2ºC respectively.

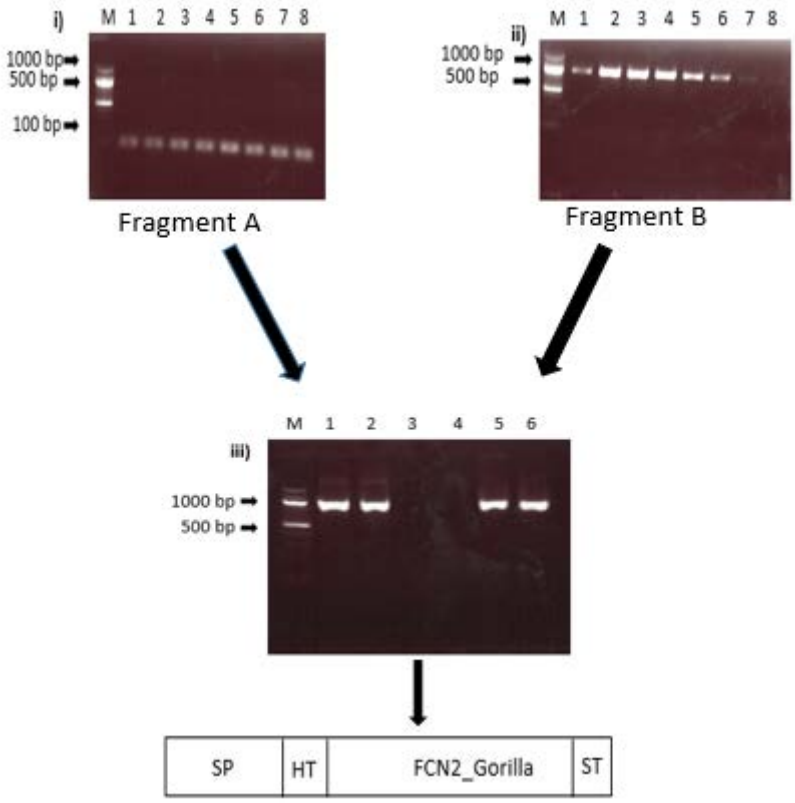

Figure 2-9: Gorilla ficolin fusion PCR products:

Fragment A (lane 1-8) (i); using gorilla ficolin forward and His-tag antisense primers by gradient PCR. Fragment B (lane 1-8) ii) using His-tag gorilla ficolin sense and Strep-tag gorilla antisense primers. The expected sizes of gorilla ficolin were detected at 97 bp (Fragment A) and 918 bp (Fragment B). The second round PCR was a combination of fragment A and B (iii) where lane 1 was with the Strep-tag gorilla ficolin antisense primer and lane 2 was without the Strep tag at an annealing temperature of 58^{o}C with negative and positive controls at lane 3 & 4 and 5 & 6, respectively. The DNA size marker (M) is shown in base pairs (bp). Lane 1-8 represents annealing temperatures of 60.9^{o}C, 62.0^{o}C, 63.4^{o}C, 65.4^{o}C, 65.2^{o}C, 67.2^{o}C, 68.9^{o}C, and 70.2^{o}C respectively.

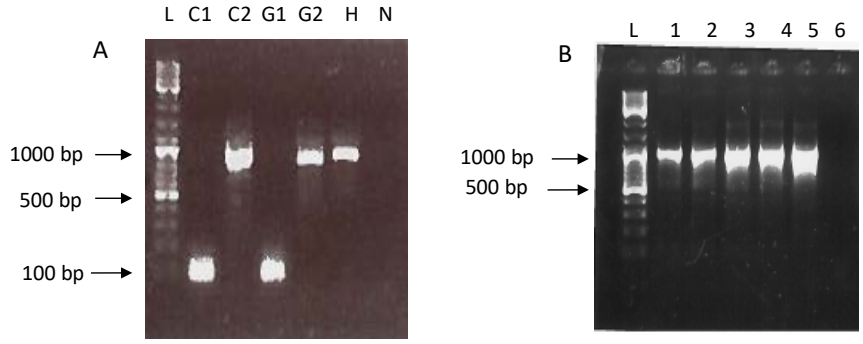

Figure 2-10 Optimising the generation of His and Strep-tagged NHP ficolin by fusion PCR

Gel electrophoresis of the resulting PCR products. A) First round PCR products; fragment A (lane C1 and G1) and fragment B (lane C2 and G2) were produced using both gorilla and colobus His-tag sense and His-tag/Strep-tag antisense primers. H is human ficolin-2 (positive control) and N is negative control with no template. B) Second round PCR product; combination of fragment A and B act as primers and templates to each other to produce the final N-terminal His-tagged and C-terminal Strep-tagged NHP ficolin-2 inserts as shown in lane 1-4. Lane 5 is human ficolin-2 as a positive control while; lane 6 is the negative control. The DNA marker is marked as L, shown on the left in base pairs.

2.3.2.2 Generation of expression clones

The NHP ficolin inserts were cloned into the pcDNA3.1D/V5-His-TOPO vector for the expression of the recombinant His-tagged NHP ficolin. They were transformed into TOP10 *E.coli,* which were plated onto ampicillin-containing agar petri dishes. Colonies were selected and positive transformants (Figure 2-11) were confirmed using the insert forward (FCN2SPCG1_F (P1)) and vector reverse (BGH) primers. These are orientation specific primers as the insert primer binds to the insert and BGH binds to the plasmid. Lane 5 (colobus, Figure 2-11 A) and lane 9 (Figure 2-11 B) were sequenced, purified and confirmed as the expression clones for NHP ficolins.

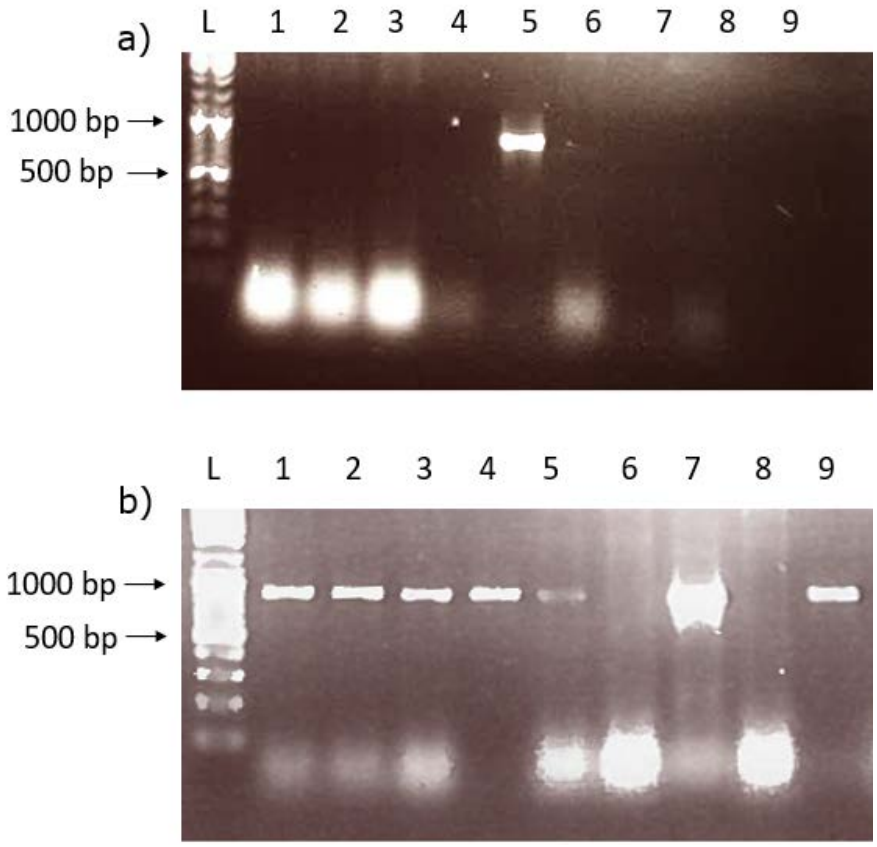

Figure 2-11: Directional PCR for the positive transformants with pcDNA3.1D/V5-His-TOPO

Directional PCR for 18 colonies of transformed TOP 10 *E.coli* growing on an ampicillin-containing LB agar plate using; insert specific forward and BGH reverse primers. The NHP ficolin insert is confirmed at 997 bp in a) colobus (lane 5) and b) gorilla (lane 1-5 and 7 and 9).

2.3.3 Phylogenetic analysis

2.3.3.1 Phylogenetic tree construction

To understand the evolutionary traits of the ficolin genes from different mammalian species, a phylogenetic tree was constructed using the maximum likelihood method based on amino acid sequences from NCBI. The sequences were aligned using ClustalW with default settings in MEGA 7 and the tree was bootstrapped 1000 times. A representative tree of all the outputs was constructed (Figure 2-12). It was shown that a group of different types of ficolins form independent clades; some were supported by high bootstrap values and some were not. This suggests that during

evolution, the predecessor of the ficolin gene has undergone duplication and each gene duplication occurred before speciation of primates and humans approximately 6 to 8 million years ago. Interestingly, the tree shows that NHP FCN2 are more closely related to the human ficolin-2 as compared to the mouse ficolins (Figure 2-12).

2.3.3.2 Amino acid sequence homology of mammalian ficolins

The pair-wise alignment comparison of the mammalian ficolins was analysed to illustrate the amino acid homology (Figure 2-13). The alignment suggests that human FCN1 is found in rat and mouse ficolin B with identities of 74% and 71% respectively. Alignment of the human ficolin-1 protein with ficolins from other species confirmed that FCN1 is present in other species such as mouse (FCNB), rat (FCNB) and primates (FCN1) as all these ficolins show high amino acid homology (at least >70%). The marmoset FCN1 seems to be an outlier amongst other FCN1 species. This is evidenced by the differences observed in the marmoset *FCN1* gene which has two alleles with insertion causing a reading frame shift [11]. On the other hand, orthologues of human ficolin-2 were only present in primates with high amino acid homology (>90%) (Figure 2-13). Human FCN3 is only found in primates, the FCN3 found in both mouse and rat have been identified as pseudogenes. In summary, ficolins with high amino acid homology might have a similar function when expressed.

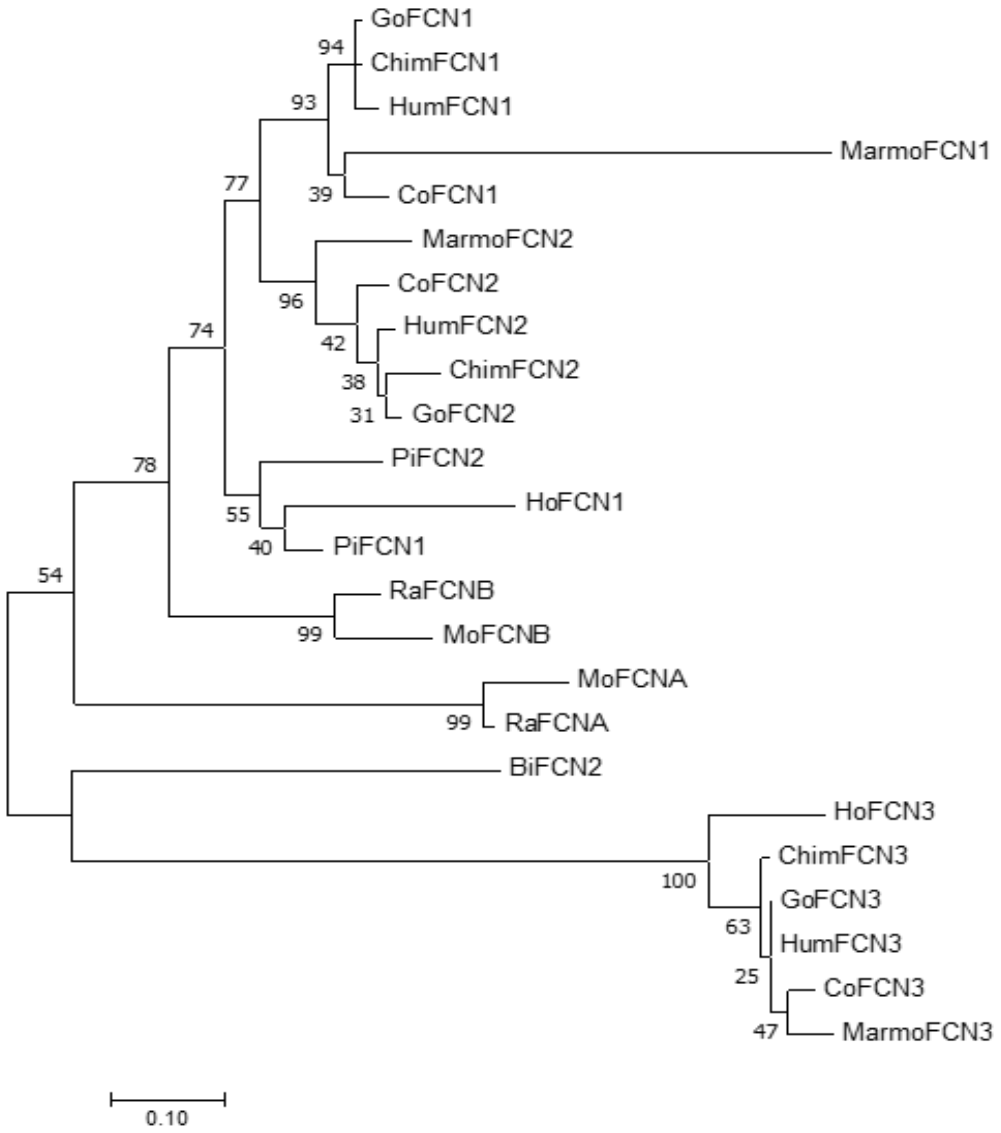

Figure 2-12 Maximum-Likelihood phylogenetic tree showing relationship of the mammalian ficolin protein family

The maximum likelihood tree was bootstrapped 1000 times based on the amino acid sequence using the ClustalW programme. The numbers on the branches represent bootstrap percentages with cut-off value of 50%.

Go=gorilla, Chim=chimpanzee, Hum=human, Co=colobus, Pi=pig, Ho= horse, Ra= rat, Mo= mouse, Bi=bird and Mar = marmoset.

	HumFCN1	HumFCN2	HumFCN3	GoFCN1	GoFCN2	GoFCN3	CoFCN1	CoFCN2	CoFCN3	HoFCN1	HoFCN3	MoFCNA	MoFCNB	RaFCNA	RaFCNB	BiFCN2
HumFCN1	ID															
HumFCN2	76	ID														
HumFCN3	42	45	ID													
GoFCN1	96	75	44	ID												
GoFCN2	75	98	46	74	ID											
GoFCN3	41	44	95	43	45	ID										
CoFCN1	92	76	43	90	76	42	ID									
CoFCN2	76	93	45	75	93	44	76	ID								
CoFCN3	43	45	90	44	46	87	43	45	ID							
HoFCN1	60	54	35	60	54	34	58	53	35	ID						
HoFCN3	43	47	84	44	47	80	43	46	82	35.6	ID					
MoFCNA	56	55	44	57	55	44	55	55	43	40	43	ID				
MoFCNB	71	65	43	74	65	43	71	66	43	51	44	56	ID			
RaFCNA	56	55	43	57	55	42	55	55	42	39	42	88	56	ID		
RaFCNB	74	68	43	76	68	42	73	68	43	54	44	57	86	57	ID	
BiFCN2	56	57	43	57	57	42	55	57	44	41	43	50	54	52	55	ID

Figure 2-13: Pair-wise percentage comparison of mammalian ficolin family

The identity of the pair-wise alignment of the amino acid sequences of ficolins expressed by different mammalian species was investigated. ID = identical; Hum = human Go-gorilla; Co=colobus; Mo=mouse, ho=horse; Ra= rat; Bi=bird.

2.3.3.3 Synonymous and non-synonymous substitutions

The coding region of the nucleotide sequence of the specific mammalian ficolin of interest (human ficolin-2, NHP ficolin-2, and mouse FCNA and FCNB) were aligned, see Figure 2-14. Table 2-5 and Table 2-6 illustrate the distances at synonymous and non-synonymous sites (dS and dN) among the 6 mammalian ficolin sequences. Generally, dN changes the amino acid while dS leave the amino acid sequence unchanged. The dN values were low within all the ficolin sequence pair-wise comparison showing a constraint on the amino acid substitutions. The dS measured between the human and primate ficolins were low, while that between the mouse ficolins and other ficolins were approximately 1 and above. The higher dS values of mouse FCNA demonstrates that amino acid variations have accumulated over time hence these sequences are different with lots of synonymous substitutions.

The dN/dS ratio was measured as this is widely used to measure the type of selection acting on a protein. Strong positive selection was indicated by dN/dS > 1, while dN/dS

< 1 indicates a negative selection. A dN/dS ratio = 1 indicated a neutral selection. Within these mammalian ficolins, a negative selection deduced from dN/dS <1 was observed (Table 2-6).

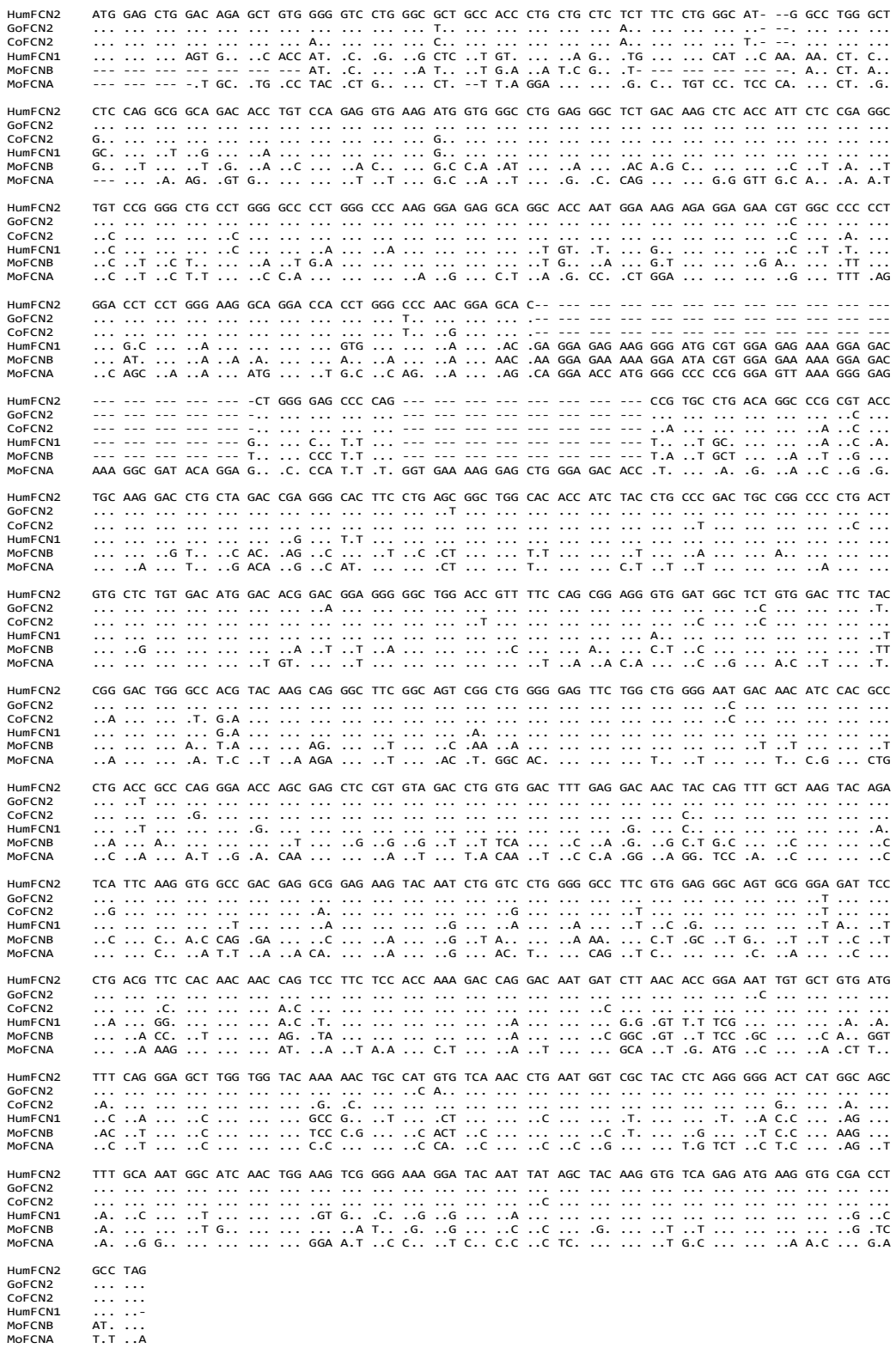

Figure 2-14: Alignment of nucleotides of the cDNA coding regions of different ficolins of different mammalian species. Dots indicate nucleotide identity with human ficolin-2 sequence. Dashes indicate gaps in the alignment.

Table 2-5: dS, upper diagonal and dN (lower diagonal) distances between mammalian ficolin sequences

	HumFCN2	GoFCN2	CoFCN2	HumFCN1	MoFCNB	MoFCNA
HumFCN2		0.042	0.095	0.302	0.998	1.215
GoFCN2	0.007		0.091	0.308	0.983	1.227
CoFCN2	0.029	0.032		0.326	1.029	1.356
HumFCN1	0.11	0.118	0.106		0.856	1.462
MoFCNB	0.192	0.191	0.19	0.162		1.179
MoFCNA	0.283	0.282	0.279	0.293	0.306	

Table 2-6: Calculated dN/dS ratio between mammalian ficolin sequences

	HumFCN2	GoFCN2	CoFCN2	HumFCN1	MoFCNB	MoFCNA
HumFCN2		0.167	0.305	0.364	0.192	0.232
GoFCN2			0.352	0.383	0.194	0.23
CoFCN2				0.325	0.184	0.205
HumFCN1					0.189	0.2
MoFCNB						0.26
MoFCNA						

2.3.3.4 Amino acid variations in the mammalian ficolin structure

To assess the possible functional implications such as oligomerisation and binding, the variations at the amino acid level in the mammalian ficolins were investigated. The amino acid sequences of primate and mouse ficolins were analysed in relation to the available data for the structure and activity of human ficolin-2. These sequences were divided into three structural regions (Figure 2-15): a signal peptide, collagen-like domain, and the fibrinogen-like domain. The signal peptide was not completely conserved among the different ficolins from different species, while the collagen-like domain was highly conserved amongst human and non-human primate ficolins. The fibrinogen-like domain at the C-terminus showed high conservation between the human ficolin-2 and NHP ficolin-2. The alignment of the amino acid diversity between different ficolin genes (Figure 2-15) revealed that the mammalian ficolins selected are unique proteins when compared at the exon level as highlighted by a rectangle;

mouse FCNA (Exons 4-6), mouse FCNB (Exon 4 & 5), human and primate FCN2 (exon 4). Although mouse FCNB seems to correspond to human FCN1 in its structural organisation.

The human ficolin-2 fibrinogen domain at the C-terminus was investigated in more detail and compared to the other species. This was like the other ficolins at different percentages; gorilla FCN2 98.5%, colobus FCN2 94.3%, mouse FCNA 69.6%, mouse FCNB 75.8% and human FCN1 84.3%. In this domain, there were several mutations between the human ficolin-2 and mouse ficolins (FCNA and FCNB) that might affect the structure and function of the proteins. The highlighted blue boxes (Figure 2-16) are significant SNPs that affect the structure and ligand binding specificities of human ficolin-2 [18]. Interestingly, the SNPs highlighted with red arrows (Figure 2-16); R- (Arg147), R- (Arg157) T- (Thr236) and A- (Ala258) are conserved in all the mammalian ficolins aligned and these affect ligand binding in human ficolin-2.

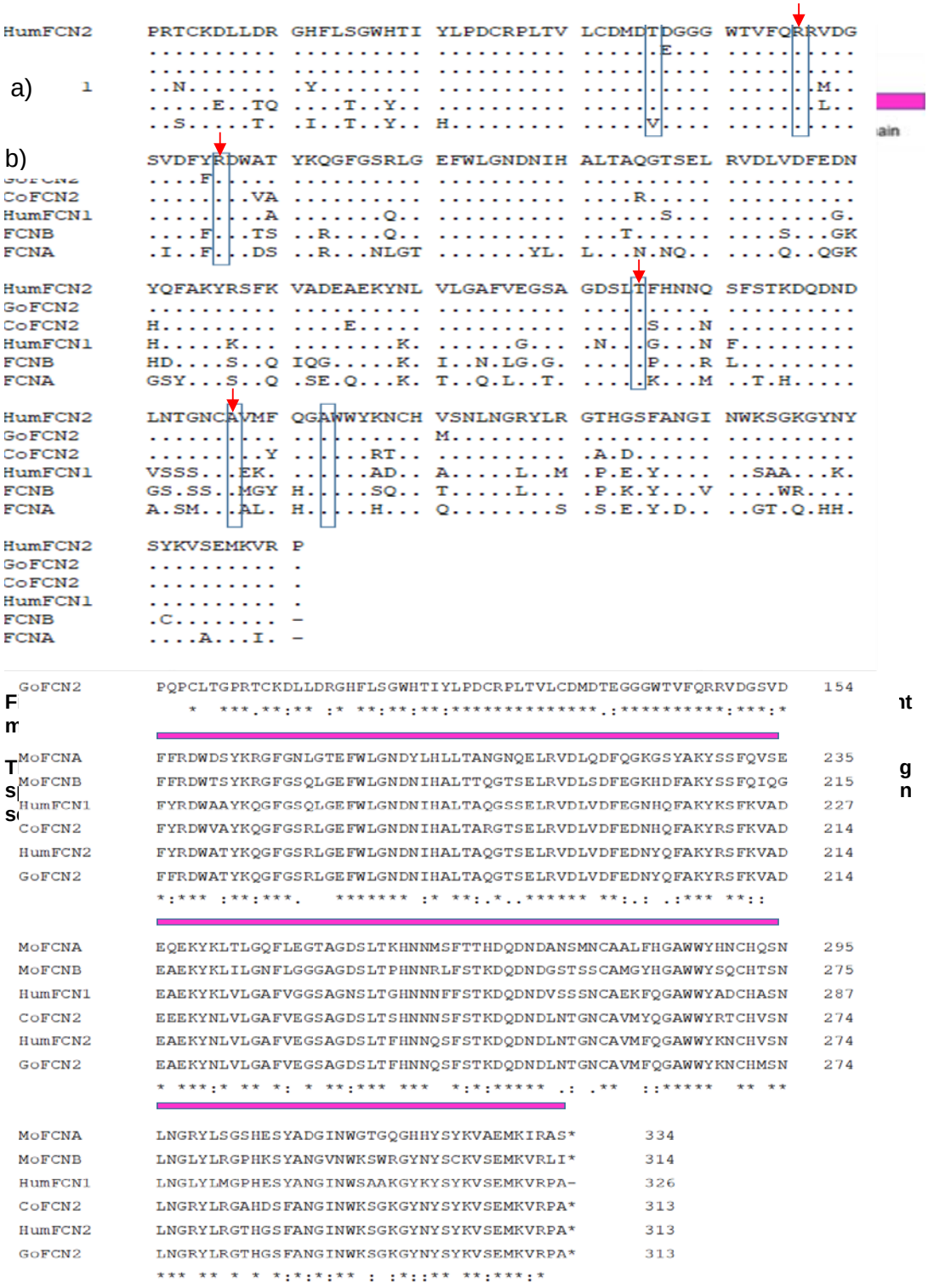

Figure 2-15: Organisation of ficolins and the amino acid diversity between different species of ficolin genes

a) functional domains of ficolins; signal peptide, cysteine region, collagen-like domain, linker and the fibrinogen-like (FBG) domain. b) amino acid sequence alignment * (asterisk) = fully conserved residue: (colon) = positions in which the amino acids have similar properties and (period) = the hydropathy of the amino acid have been preserved in the course of evolution. The box shows the exon difference in the mammalian ficolins despite their conservation.

2.3.3.5 Crystal structure showing variation and conservation of the FBG domain

The conservation residues of the mammalian ficolins were mapped onto the human ficolin-2 FBG domain structure (Figure 2-17). The trimeric structure indicates that each monomer is showing conservation and variation (Chain A), the binding sites; S1-S4 (Chain B) and the Ca^{2+} ion (Chain C). Chain A indicates conserved amino acids represented in mauve and the highly variable amino acids in cyan. The conserved patches on chain A correspond approximately to binding sites 2, 3 and 4, while the variable sites approximately correspond to binding sites 1 and part of 2. This shows that the highly conserved site is slowly evolving and retaining its binding site while the variable sites are rapidly evolving leading to a selective pressure. This might lead to the ficolin expressed by different mammalian species to recognise different pathogens in different ways.

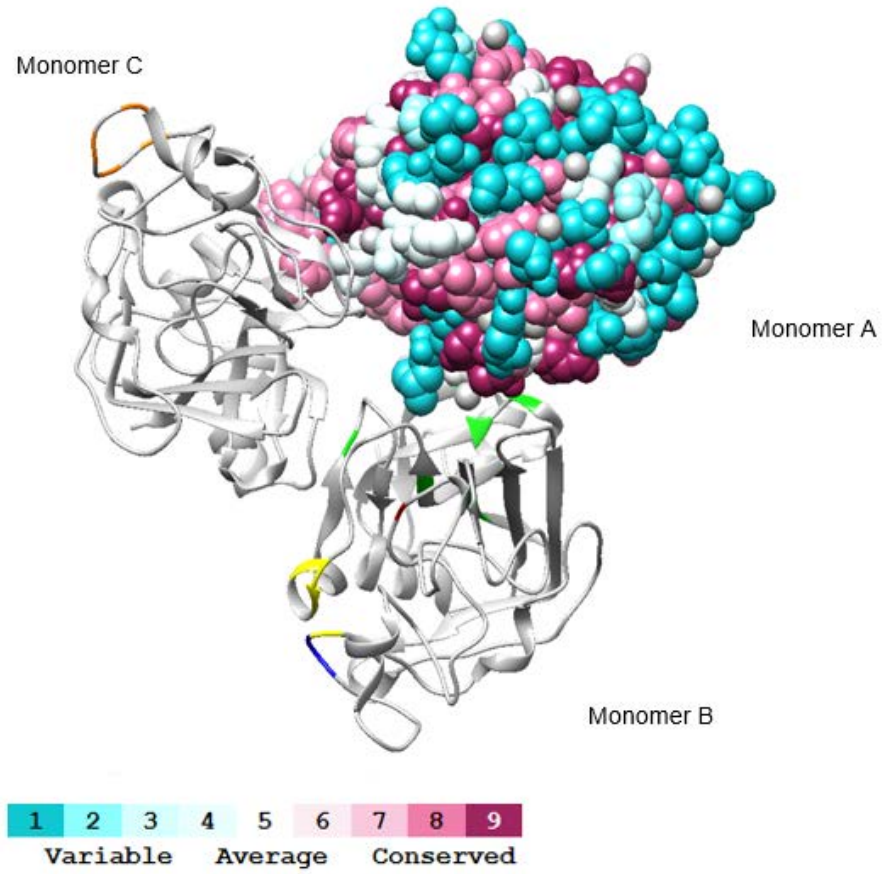

Figure 2-17: Conservation and variability in the mammalian ficolin FBG domain mapped onto PDB 2j3g using the Consurf server

Homotrimeric structure of ficolin recognition domain (FBG domain) showing conservation and variability plots. Conservation plot was generated using an alignment of 8 ficolins amino acid sequences manually inputted. Alignments were plotted using the Consurf server [274], mapped unto PDB 2j3g. Monomer A shows conservation (mauve) and variation (cyan) while B (binding sites highlighted) and C (Ca^{2+}) are ribbon diagram of the other 2 monomers. The level of conservation is measured with a range of 1-9 with 9 being the highest as shown by the key.

2.4 **Discussion**

Ficolin genes have been identified in different mammalian species including humans [5, 6, 278], NHP [11] and mice [9]. In humans and NHP, three ficolin genes, *FCN1, FCN2* and *FCN3* have been identified while *fcna* and *fcnb* are found in rats and mice. *FCN3* in both rat and mouse ficolin is a pseudogene [19]. Although there are limited studies on the production of recombinant NHP ficolin, NHP ficolin genes have been

characterised to be similar to the human ficolin genes known [11]. In order to produce NHP FCN2 constructs, liver samples were collected from a colobus monkey, a marmoset and a gorilla. Subsequently, total RNA was isolated, cDNA reverse transcribed from mRNA and used as a template for PCR. The quality of the RNA was confirmed by detecting mRNA expression of the GAPDH gene (Figure 2-3). GAPDH is a housekeeping gene frequently used as an endogenous control and has been shown to have consistent expression and can be used in numerous experiments [279]. Double-tagged recombinant colobus and gorilla FCN2 were produced. The N-terminal His-tagged ficolin was created by fusion PCR (Figure 2-2) which was inserted downstream of the peptide cleavage site to enable its detection using an anti-His-tag antibody while a Strep-tag was attached to the C-terminus. An N-terminal His-tagged NHP FCN2 would avoid hindering the effect of the C-terminal fibrinogen domain, responsible for ligand binding. Although the N-terminal is more flexible, it contains a cysteine-rich region and the collagen domain responsible for the formation of trimeric subunits into active oligomers [280]. This oligomerisation is important in the functionality of all ficolins. Hence, the double tag should possibly enable highly purified and homogenous protein preparations.

Except for a recent qualitative de novo analysis of full-length cDNA of marmoset transcriptomes [281], this is the first time a full-length coding region of the NHP ficolin gene has been amplified. Human ficolin-2 was detected in the presence of non-specific bands and primer-dimer (Figure 2-4) showing successful amplification. Gradient PCR was used to identify the best annealing temperature without affecting the amplification quality of the DNA. Two products persisted in all the samples except for plasmid control. To resolve the non-specific bands; an additional primer set was designed to avoid regions with high self-complementarity and priming of unintended regions. The sequencing of colobus and gorilla FCN2 was carried out as they had more prominent bands expected (Figure 2-7). Interestingly, sequence data analysed on the NCBI BLAST server showed the similarity between NHP FCN2 and other NHP

ficolins deposited on Genbank. However, there was no 100% matching sequence, showing that the NHP sequences had some mismatches. After the successful cloning of the NHP FCN2, the sequenced data was further analysed.

The phylogenetic relationships of ficolins from different mammalian species were explored by generating a maximum likelihood tree using protein sequences from NCBI (Figure 2-12). For some ficolins (human, gorilla, colobus, marmoset and chimpanzee ficolin-2), a cluster was found, suggesting that they all might have evolved from a common ancestor. However, marmoset ficolin-1 seems to have diverged differently. This is consistent with another study where a significantly different allele of the *FCN1* gene was detected in the common marmoset [11]. In the phylogenetic tree generated (Figure 2-12), *FCN2* gene is seen in primates and not mice (which has a *fcna* or *fcnb* gene) proving their divergence. They might have branched out by gene duplication. The molecular clock hypothesis states that protein sequences evolve at a rate relative to the time when the divergence of two species is compared [282, 283]. Gene function changes over time [284] while mutational and DNA repair mechanisms vary among different organisms [285], hence no protein would evolve at a constant rate for a long evolutionary time. If the molecular clock hypothesis holds true, it is possible to use the hypothesis to estimate divergence timescales. Although the divergence times were not calculated in this study, several methods have been used to estimate the divergence times for few mammalian and several primate species [286] and this was inferred from phylogenetic trees. Studies have estimated that the divergence times between human and mice and between human and gorilla are about 96 and 7 million years (MY) ago, respectively. This might suggest why the orthologue of human ficolin-2 was only observed in NHPs. The pig ficolin also showed an independent duplication of their gene. It is plausible to conclude that ficolins from different mammalian species have duplicated their first gene independently, and this might be because of selective pressure on each gene within a period.

The phylogenetic tree suggests that the ficolin precursor has undergone an expansion which has involved an independent duplication of events during evolution. This is consistent with observations made when the genetics of ficolins from both vertebrates and invertebrates were explored as reviewed in Chapter 1 [17, 287]. In contrast to the current study, more ficolins were studied, and this gave more insights on the origin of each ficolin and their source of evolutionary divergence [17]. Interestingly, this study also proved the high homology of the exon organisation of ficolins (Figure 2-15 B). From the alignment of the amino acid sequences, mouse FCNA and FCNB, when compared to the human and NHP FCN2, vary greatly in their exon organisation as highlighted with a rectangle (Figure 2-15). This difference is in exons 4-6 in mouse FCNA and corresponds to exon 4 in human ficolin-2 and exon 4 and 5 in mouse FCNB and human FCN1. Collectively, mouse FCNB and human FCN1 are orthologues while mouse FCNA and human ficolin-2 are different proteins. This observation is consistent with another study where the genetics of ficolins were studied [17]. Authors suggested that FCN1 is an orthologue which might have been duplicated in different mammalian species. This proves that these proteins are different and might manifest varying binding capabilities. To further support the observations above, the pair-wise alignment of the amino acid sequence was investigated. More than 70% amino acid consensus sequences of different mammalian ficolins were found using pair-wise alignment (Figure 2-13). The orthologues of each type of ficolin were aligned, but the percentages were low compared to the analysis made by Garred, P., et al., [17] which excluded the signal peptides. This may be justified, as the signal peptide of the ficolin from different mammalian species is not completely conserved.

The evolution of ficolins from selected mammalian species did not show any positive selective pressure. The same was observed when the evolution of the mannose-binding lectin gene in primates was characterised [288]. Hence, there is no evidence that the direct interaction of mammalian ficolins with different pathogens promote selective pressure. To study the variations at the amino acid level, the different

domains of the ficolin molecule were examined using Clustal W Omega (Figure 2-15). The signal peptide is usually 16-30 amino acid residues responsible for directing a protein to the rough endoplasmic reticulum [289]. The signal peptide of the mouse ficolins had a shorter signal peptide of about 16 amino acids compared with human and NHP ficolin-2 which had about 25 to 27 amino acids. Interestingly, all the mammalian ficolin signal peptides seem to contain many hydrophobic amino acids which suggested that they had maintained their hydrophobicity; this is important for bonding with the signal recognition particles (SRPs). The collagen-like domain enables the oligomerization of ficolins and modulation of an immune response through interactions with MASPs [6, 13]. The role of this domain explains its high conservation amongst the human and NHP FCN2 but variable in mouse ficolins.

The C-terminal fibrinogen-like domain (FBG) is the most interesting domain, as it is responsible for the binding of ficolin to PAMPs. The non-synonymous variations are mostly clustered in this region as highlighted (Figure 2-16) and this might affect their binding specificity to different viruses. During evolution, the FBG domain of ficolins from different mammalian species might co-evolve with viruses and be modified, giving rise to a broader diversity in their recognition [11]. SNPs in human ficolin genes are non-synonymous substitutions [72, 73]. Interestingly, there is an obvious similarity when comparing the human ficolin-2 non-synonymous variation with other mammalian species (Table 2-5). This is consistent with other variations in human ficolin-2 [73, 79-81]. It is noteworthy that non-synonymous variations affect ligand binding; Arg147Gln and Arg157Gln [73]; which might affect the binding of ficolins from different mammalian species to hepacivirus glycoproteins. The crystal structure of the FBG human ficolin-2 and its interaction with different ligands has been resolved [12]. This allowed the conservation and variability of the different binding sites in the FBG to be mapped (Figure 2-17) on the human ficolin-2 model with PDB ID 2j3g. The extracellular part of the FBG domain is more variable because of interaction with different pathogens. This provided more evidence of the specific mutations seen when

the amino acid sequences were aligned (Figure 2-16). This could be explained by the adaptation in the exposure to different pathogens for different ficolin species. Hence the mammalian ficolins might bind differently.

2.5 **Conclusion**

In conclusion, ficolins from different mammalian species have homology with different binding characteristics. This chapter described initial steps in cloning to analyse ficolin proteins isolated in different primate species. Expression clones of FCN2 from non-human primates was achieved which will enable the production of recombinant ficolins from different mammalian species. The successfully expressed proteins will enable investigation of their ability to bind to E1/E2 glycoproteins. The phylogenetic tree analysis revealed a tight cluster between the different ficolin species. However, most non-synonymous variations seem to cluster in the FBG domain and thus affect affinity or specificity of ficolin binding to different viruses. The FBG domain may have co-evolved with pathogens, hence modified during evolution. This modification may influence a broader diversity against different pathogens.

3 Production, Purification and Binding Specificity of Ficolin proteins Expressed by Different Mammalian Species

3.1 Aims

This part of the investigation aimed to study the production and purification of recombinant ficolins expressed in different mammalian species. Expression and purification strategies for human, primate and mouse ficolins were developed. The direct antiviral phenotype of ficolins recovered from species other than humans have not been studied extensively. Therefore, it was important to characterise the function of these proteins by expressing them and confirming their ability to bind to their specific ligands. This study also included the characterisation of both purified and unpurified recombinant ficolins generated using *in vitro* expression systems, before determining the interaction of these proteins with the glycoproteins of hepatitis C virus. The viral glycoproteins E1 and E2 were subsequently produced in order to characterise their interactions with ficolins. Ficolins recovered from both human and non-human species were used to analyse species-specific differences in glycoprotein interaction.

3.2 Materials and Methods

3.2.1 Antibodies and Reagents

Unless otherwise specified, all reagents used were commercially purchased from Sigma Aldrich Ltd, UK and are as follows; Sodium dodecyl sulphate (SDS), para-nitrophenyl phosphate substrate (pNPP), molecular grade water, kanamycin, ammonium persulphate (APS) Tween 20, ampicillin, calcium chloride ($CaCl_2$), sodium chloride (NaCl), imidazole, ethidium bromide, 2-mercaptoethanol and dithiothreitol (DTT). Reagents used in cell culture were; phosphate-buffered saline (PBS), trypsin/EDTA, dimethyl sulfoxide (DMSO), non-essential amino acid (NEAA) and

heat-inactivated fetal bovine serum (FBS; Gibco). Antibodies used are listed in Table 3-1. Anti-His tag antibodies were purchased from ThermoFisher and Abcam, while secondary antibodies were purchased from Dako and Sigma. Mouse monoclonal antibodies to HCV glycoprotein E2 (ALP98 and AP33) [290] were a kind gift from Dr Arvind Patel (Centre for Virus Research, Glasgow).

Table 3-1 Antibodies used in this study

	Primary Antibody	**Secondary Antibody**
His-tagged Ficolins	6X his epitope tag antibody (His.H8) (Thermo Scientific) Monoclonal 6X His-tag 4D11 (Abcam) GN5, Mouse anti-human ficolin-2 monoclonal antibody	Rabbit anti-mouse immunoglobulins horseradish peroxidase (HRP) antibody (Dako)
Anti HCV	Anti-HCV E2 ALP98 (Mouse), Anti-HCV E2 AP33 (a gift of Arvind Patel)	Anti-Mouse IgG (Fc Specific) Alkaline Phosphatase (AP) Conjugate (Sigma)

3.2.2 Plasmids and Bacterial Strains

The non-human primate ficolin-encoding genes were cloned into the expression vector pcDNA3.1D/V5-His-TOPO (Invitrogen) in the previous chapter. Commercially available plasmid expression vectors expressing mouse ficolins FCNA and FCNB in pCMV3-SP-N-His were obtained from Sino Biological Inc. All plasmid stocks were transformed in One Shot™ TOP10 chemically competent *Escherichia coli (E. coli)* (C4040-03; Thermo) and were grown aerobically at 37°C overnight (O/N). Positive clones were cultured in Lysogeny Broth (LB) medium containing ampicillin (100 $\mu g.mL^{-1}$) or kanamycin (50 $\mu g.mL^{-1}$).

3.2.3 Cell lines, media and Kits

Human Embryonic Kidney Cells (HEK 293T) expressing the SV40 T antigen (kindly provided by the MRC-University of Glasgow Centre for Virus Research) were used for transfection with plasmids encoding His-tagged ficolins. Cells were cultured in

Dulbecco's Modified Eagles Medium (DMEM) or Opti-MEM medium (GIBCO BRL, Paisley UK). DMEM was supplemented with 10% FBS and 0.1 mM non-essential amino acids (NEAA). Cells were maintained in a 75 cm^2 or 225 cm^2 Corning tissue culture-treated flasks with vented caps. Subsequently, cells were passaged when they reached 90% confluence at a density of 5-9 x 10^6/mL using trypsinisation (0.05% Trypsin/1mM EDTA; Gibco) (approximately 72 -96 hrs after seeding). Kits used were; BCA Protein Assay Kit (Pierce/Thermo Scientific) and Endotoxin-free High Purity Midiprep or miniprep kit (Sigma).

3.2.4 Mouse ficolin plasmid construction

Mouse ficolin (FCNA and FCNB) expression clones were transformed into TOP10 competent cells (Invitrogen) for maintenance. One microlitre of cloned plasmid was added to 25 µL of TOP10 competent *E. coli* cells thawed on ice, then mixed gently. The mixture was incubated for 30 minutes and heat- shocked at 42ºC for 30 secs in a water bath. The cells were then transferred to ice for 2 minutes, followed by addition of 250 µL Super Optimal broth with Catabolite repression (S.O.C.) media. Cells were incubated at 37ºC for one hour, after which 100-200 µL culture was plated out on Lysogeny Broth (LB) agar plates with 50 $\mu g.mL^{-1}$ kanamycin at 37ºC overnight. Positive clones were cultured in 7 mL or 50 mL LB containing 50 $\mu g.mL^{-1}$ kanamycin overnight.

3.2.5 Ficolin plasmid purification and recovery

All ficolin-encoding plasmids were purified using GenElute™ Plasmid Miniprep or MidiPrep kits (Sigma) following the manufacturer's protocols. The plasmid was eluted in 10 mM Tris-HCl at pH 8.5. The purity of the DNA was quantified using a Nanodrop spectrophotometer with the ND-1000 v3.7.1 software, recording absorbance at 260 nm and 280 nm**.**

3.2.6 Transfection of Human Embryonic Kidney (HEK) 293T cells with ficolin-encoding plasmids

3.2.6.1 Transient transfection of HEK 293T with plasmids encoding mouse ficolin genes

HEK 293T cells were used for the transient expression of mouse ficolin constructs. A total of 1.2 x 10^6 cells/mL HEK 293T cells were seeded prior to each transfection in a 10 cm diameter Primaria-coated tissue culture dish, using Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS medium. This resulted in 40-50% cell confluence after 16 h incubation at 37ºC in 5% CO_2. After incubation, 2 µg (2000 ng) of FCNA or FCNB plasmid were diluted in 300 µL Opti-MEM (Gibco) with 24 µL of polyethylenimine (PEI) each and incubated for 45 minutes at room temperature. For transfection, cell media was changed to 7 mL Opti-MEM (Gibco) and the DNA-PEI mix was added. A negative control transfection without an expression plasmid was included. The medium was replaced with new DMEM/FBS six hours after transfection. At 72 h post-transfection, the supernatant was harvested. This process was further optimised with cells being seeded at 2x10^6 cells per dish, and transfection was repeated to include the human ficolin-2 expression construct as a positive control.

To further optimise the expression of the mouse ficolin, increase yield and eliminate possible contamination by bovine serum albumin (BSA) in DMEM, the following variations were considered; a positive control (human ficolin-2) was included; density of cells was increased (to 1 x10^7 cells/mL); the amount of plasmid used for transfection (Figure 3-1) was increased (from 2000 ng to 6000 ng or 8000 ng); expressed protein was concentrated rather than purified; and a reduced serum medium was used throughout the transfection, including the 72 h incubation. This experiment was a pilot study for the expression of non-human primate ficolin expression in this study.

3.2.6.2 Transient transfection of HEK 293T with plasmids encoding ficolins genes from different mammalian species

HEK 293T cells were used for the transient expression of different mammalian ficolin constructs. HEK 293T cells were seeded in T175 or T225 cm^2 flask at a concentration of 5 x 10^6 or 1 x 10^7 cells/mL respectively, prior to transfection in DMEM/10% FBS medium for 40-50% confluence and incubated overnight. Murine FCNA or FCNB, gorilla ficolin-2, or human ficolin-2 plasmid at a concentration of 6000 ng or 8000 ng were diluted in 1200 µL Opti-MEM (Gibco) with 96 µL of PEI each and incubated for one hour. The media was replaced with fresh Opti-MEM before adding the DNA-PEI mix. A negative control (FCNNeg) transfection prepared in the absence of an expression plasmid was included. The medium was replaced with 7 mL Opti-MEM six hours after transfection. At 72 h post-transfection, the supernatants containing either FCNA, FCNB, FCN2 (human and gorilla) or negative control were harvested. This process was further optimised by scaling up to using 4 to 5 flasks for each plasmid, to increase the amount of protein expressed after harvesting.

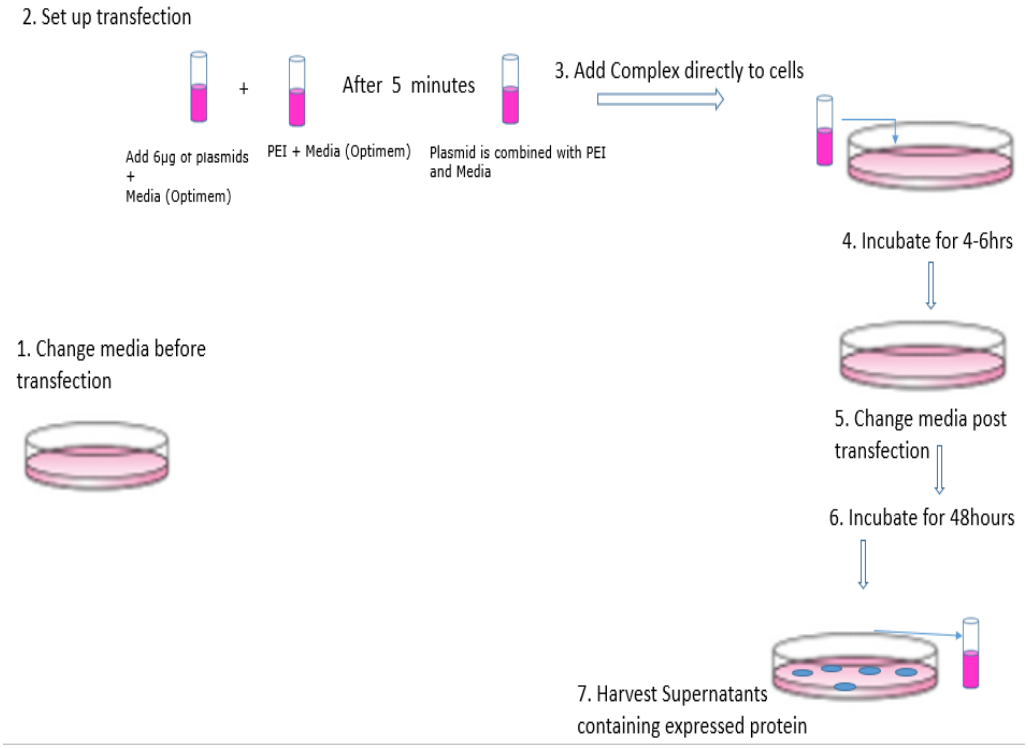

Figure 3-1 Schematic overview of transfection in HEK 293T cells

The schematic representation summarises the different steps involved in transfecting ficolin into HEK 293T cells. 1) changing the media to reduced serum media before transfection 2) Set up transfection by adding transfection reagent to plasmid and incubate 3) Add transfection complex to cells 4) Incubate for four hours 5) Replace media with full medium 6) Incubate for 72 h 7) Harvest supernatants containing expressed proteins.

3.2.7 SDS-PAGE and silver stain and Western Blot (WB) analysis of proteins

3.2.7.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein separation was achieved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS- PAGE) [291]. Precast gels (10% polyacrylamide; Bio-rad) were used. To prepare each sample, 5 µL of each protein-containing supernatant was added to 5 µL of loading buffer and heated at 95^{o}C for 5 minutes to denature proteins. Samples were loaded under non-reducing and reducing conditions by adding a reducing agent; either 5% of 2-mercaptoethanol or 20 mM DTT were added to the loading buffer as reducing agents. Proteins were electrophoretically separated in parallel with a molecular marker (Merck) at 150 V for one hour.

3.2.7.2 Silver staining of protein gels

An equal volume (10 µL) of purified samples and loading buffer were heated at 95^{o}C for 5 minutes before loading. Gels were stained with silver stain using SilverQuest stain kit Dodeca™ Silver Stain Kit (Cat. 1610480; Bio-Rad) according to the manufacturer's protocol. After staining, the gel was manually dried and imaged using a G: box imaging system (Syngene).

3.2.7.3 Western Blot

Resolved proteins were transferred to a nitrocellulose or a polyvinylidene difluoride (PVDF) membrane (Amersham) using a trans-blot semi-dry transfer cell system (Bio-Rad 200) for one hour or a Transblot Turbo system (Bio-Rad) for 30 minutes. Blotting paper and membrane were soaked in a transfer buffer (39 mM glycine, 48 mM Tris base, 20% methanol, 1.3 mM SDS at pH 8.3) prior to use.

The membrane was blocked with 5% skimmed milk and 0.05% Tween 20 in phosphate-buffered saline (PBST) overnight at 4^{o}C. Next day, the membrane was washed three times in between each stage for 5 minutes with PBST and incubated with primary antibody; anti-polyhistidine tag purified antibody (E-Bioscience) or 6X His-Tag monoclonal antibody (HIS.H8) (MA1-21315) or anti-6X His-tag antibody (4D11) at 1 µg.mL^{-1} in 5 mL of PBST. Expression of His-tagged protein was detected with the polyclonal anti-mouse immunoglobulins/Horseradish Peroxidase (HRP) (Dako) at 1:1000 in 5 mL of PBST. DTT or 2-mercaptoethanol was used as a reducing agent. Unbound proteins were removed by washing 3 times with PBST following incubation. Finally, the membrane was developed using Amersham Hyperfilm™ ECL™ high-performance chemiluminescence film (GE Healthcare) for 5 minutes or a radiance chemiluminescent substrate (Azure Biosystems) for 1 minute. The membrane was put on a clear sheet and imaged using a Syngene G: Box imaging system. The exposure time for image capture was set to a range between 5 to 10 minutes to increase the sensitivity of these assays.

3.2.8 Protein concentration

Amicon® Ultra 15 mL 10K MWCO centrifugal filter units (Sigma) were used to concentrate the protein according to the manufacturer's manual. The ultrafiltration membrane in the centrifugal unit may contain a trace of glycerine and was therefore primed with 5 mL of PBS before use. Each ficolin preparation; FCNA, FCNB, FCN2 (gorilla and human) and FCNNeg were concentrated to either 1 mL, 2 mL, 5 mL or 50 mL ready for purification. The ficolin supernatants were also concentrated to the lowest concentrate volume of 250 µL for assay optimisation.

3.2.9 Purification of the expressed protein

3.2.9.1 Immobilised Metal Affinity Chromatography (IMAC)

The polyhistidine-tagged ficolins expressed were purified by IMAC using a nickel-agarose 5 mL Histrap HP column (Cat. 17524802; SLS) on an AKTAPrime plus system. 50 mL of concentrated ficolin supernatant was used for IMAC purification. The column was equilibrated using 5-column volumes (CV) wash/binding buffer (20 mM Sodium phosphate, 0.5 M). After loading and injecting the sample, weakly bound proteins were removed with 3 CV wash buffer. Subsequently, samples were eluted with 3-5 CVs of the elution buffer (20 mM Sodium sulphate, 0.5 M imidazole and 300 mM NaCl, pH 7.4). The start material and eluates including flow-through were analysed by western blot analysis and silver stain, and the peak fractions were stored at 4^{o}C for further analysis.

3.2.9.2 MagneHis Protein Purification System

The MagneHis™ protein purification system (Cat.V8500, Promega) was used to purify the polyhistidine proteins from 1 mL of transfected HEK 293T cells, according to the manufacturer's protocol [292]. Briefly, 30 µL of the MagneHis™ Ni particles were added to 1 mL of concentrated ficolin supernatant. This was then incubated to bind for 2 minutes at room temperature and placed on a magnetic stand for approximately

30 secs. Five hundred microlitres of MagneHis™ binding/wash buffer containing 500 mM NaCl was used to remove unbound proteins, using 3 repeated washes. His-tagged proteins were eluted using 100 µL of MagneHis™ elution buffer. Additionally, 2 mL or 5 mL of the concentrated ficolin preparation were aliquoted into either two or five 1 mL eppendorf tubes respectively, and each was purified. The 2 or 5 mL, concentrated ficolin preparation, were pooled together to make 200 µL or 500 µL respectively after the first elution. A second elution was performed to release more polyhistidine ficolin protein. Both fractions were analysed for the presence of ficolins by SDS-PAGE and western blot.

3.2.10 Quantification of NHP, human and mouse ficolins

3.2.10.1 Bicinchoninic acid (BCA) protein assay

The total protein concentration was quantified in a colorimetric reaction with BCA assay [293]. A commercially purchased BCA assay kit (Thermo Scientific) was used. A series of provided BSA standards (0, 25, 125, 250, 500, 750, 1000, 1500, 2000, $\mu g.mL^{-1}$) in saline or Opti-MEM were prepared. Twenty-five microlitre of each standard or unknown sample replicate were added to a Costar® flat-bottomed, low-binding, non-sterile, 96-well plate (Corning Inc.). 200 µL of Pierce™ BCA Protein Assay reagent mix (Thermo Scientific) A (working solution) and B were added at a ratio of 50:1 (A: B). The plate was covered, mixed on a micro-shaker for 30 secs and placed in a sandwiched box with moist tissue ready for incubation. It was then incubated at 37°C for 30 minutes. The absorbance of all samples was measured at 562 nm using FLUOStar Omega plate reader. The blank was used as control, and results were analysed using GraphPad Prism 6 software (San Diego, CA, USA). Protein concentration was calculated by comparison to a standard curve.

3.2.10.2 Enzyme-linked immunosorbent assay ELISA

Maxisorp™ flat-bottomed 96 well immunoassay plates (Thermo Scientific; Cat. 442404) were coated with 50 µL of 100 µg.mL^{-1} acetylated bovine serum albumin (AcBSA) in Dulbecco's PBS. After overnight incubation at 4°C, the wells were washed with PBS 0.05% Tween (PBST). Wells were thereafter blocked with 200 µL 5% skimmed milk dissolved in PBS containing 0.05% Tween (PBSTM) for 4 h at room temperature (RT). The plate was washed with 200 µL of Tris-buffered saline-Tween-calcium (TBST-Ca) containing 0.05% Tween (v/v) and 5 mM $CaCl_2$ (wash buffer). This wash buffer was used in subsequent washes. Wells were incubated with 50 µL of ficolin A, ficolin B, human ficolin-2, gorilla ficolin-2, negative control (cells only) and serum diluted in the wash buffer at 4°C overnight. After this incubation, the plates were washed 3 times as previously described. For the detection of all ficolins expressed, 50 µL of anti-6X His-tag antibody or GN5 antibodies (1 µg.mL^{-1} & 100 µg.mL^{-1}), or anti-ficolin-2 antibody (FCN2-K19) were added to each well, respectively. The plates were incubated for 2 h at RT. Wells were washed and incubated for 30 minutes at 37°C with anti-mouse-IgG conjugated to alkaline phosphatase (AP) (Sigma) diluted at 1:1000 in the wash buffer. Following another three washes with 100 µL of wash buffer, binding was visualised with 100 µL of pNPP substrate (Sigma). The absorbance was measured at 405 nm using a FLUOstar Omega microplate reader after 30 minutes.

3.2.11 Acetylated bovine serum albumin (AcBSA) and bovine serum albumin (BSA) Binding Assay

Maxisorp™flat bottomed 96 well plate (Thermo Scientific; Cat. 442404) was coated with 50 µL of 100 µg.mL^{-1} acetylated bovine serum albumin (AcBSA) in Dulbecco's PBS, or 4 % BSA. After O/N incubation at 4°C, the wells were washed with PBST. Residual binding sites were blocked with 200 µL of PBS 0.05% Tween- (v/v), 5% milk

(w/v) (PBST-M) for 4 h at room temperature (RT). The plate was washed with 200 µL of TBST (Tris-buffered saline-Tween) containing 0.05% Tween (v/v).

To assess whether expressed mammalian ficolins exhibited calcium-dependent binding to AcBSA, 50µL of FCNA, FCNB, HumFCN2, GoFCN2 and negative control were diluted in TBS/Tween or TBST-Ca with 0.5 mM, 5 mM and 50 mM of $CaCl_2$ or EDTA and incubated at 4°C overnight. The binding/wash buffer for the calcium and EDTA assay contained no calcium (TBST) and 5 mM calcium (TBST-Ca) respectively. After this incubation, the plates were washed 3 times with TBST or TBST-Ca and incubated with 50 µL of anti-6X His-tag antibody (Abcam Ab5000 or Thermofisher MA 21315) at 1 $\mu g.mL^{-1}$. The plates were incubated for 2 h at RT. Wells were washed and incubated for 30 minutes at 37°C with a 1:1000 dilution of anti-mouse IgG-alkaline phosphatase (AP) in the wash buffer. Following another three washes with 100 µL of wash buffer, binding was visualised with 100 µL of p- nitrophenyl phosphate substrate (pNPP; Sigma). The absorbance was measured at 405 nm using a FLUOstar Omega microplate reader after 30 minutes.

To assess the specificity of ficolin binding to AcBSA or BSA, 10-fold serial dilution from 1/10 to 1/1280 of the ficolin-containing preparations were prepared in TBS/0.05% Tween-20/ 5 mM $CaCl_2$. TBS/Tw/$CaCl_2$ was used as a washing buffer for the rest of the experiment. Subsequently, wells were incubated with primary and secondary antibodies as mentioned above. Then, pNPP was added, and the absorbance was measured at 405 nm using a BMG Labtech FLUOstar OMEGA plate reader.

3.2.12 Binding of ficolin with HCV glycoprotein

3.2.12.1 HCV glycoprotein expression

A full-length HCV E1/E2 glycoprotein clone from genotype 1 isolate H77c with amino acid residues 170-746 ($E1E2_{170-746}$) was previously developed by Dr Alexander Tarr

and has been well characterised [294-297]. This clone was subcloned into pcDNA3.1 mammalian vector and ready for transfection. The plasmid encoding the full-length E1/E2 glycoproteins were purified using Midiprep kits as described in section 3.2.5 and subsequently transfected into cells. HEK 293T cells was seeded at a density of 1.2 x 10^6 cells/mL in DMEM in tissue culture 10 cm diameter Primaria-coated sterile dishes (Corning) overnight prior to transfection to achieve ~60% confluence. The media supporting these cells was replaced with 7 mL fresh Opti-MEM whilst preparing the DNA-Medium-PEI mix. Two reaction mixtures were prepared as follows; a) 2000 ng plasmid in 300 µL Opti-MEM; and b) 24 µL PEI in 300 µL Opti-MEM. Reaction mix B was incubated for 5 minutes and added to mix A. After mixing the plasmid-PEI solution, it was incubated for 1 h at room temperature and added in a drop-wise manner to the cells. The cell-associated E1/E2 was harvested 72 h post-transfection by lysing cells with lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% IgePal-650, 20 mM iodoacetamide, pH 7.6). To each plate, 1 mL of lysis buffer was added and placed on a plate shaker for 15 minutes at 10 rpm at room temperature. Subsequently, lysates were centrifuged at 8,000 x g for 5 minutes and the supernatant was used as a source of E1/E2 and analysed by SDS-PAGE (10% polyacrylamide gel). The remainder was stored at -20^{o}C for further analysis.

3.2.12.2 SDS-PAGE and Western Blot Analysis

The expressed E1/E2 glycoproteins were analysed by separation using gel electrophoresis, as detailed in Chapter 3 section 3.2.7.1. Western blotting was used to identify the expressed E1/E2 as described in section 3.2.7.3. The membrane was washed 3 times for 5 minutes in PBS-0.05% Tween after blocking and incubated in the primary antibody directed against E2, ALP98 [290] (1/500 in PBST-0.05% Tween) overnight. Thereafter, the membrane was incubated with HRP-conjugated rabbit anti-mouse antibody (Dako) as a secondary antibody for 1 h. The wash step was repeated after each step. The protein was visualised using an imaging system detailed in section 3.2.7.3.

3.2.12.3 Ficolins and HCV glycoprotein binding assay

Nunc™ MaxiSorp™ flat-bottomed 96-well plates (Thermo) were coated with 50 µL of anti E2 antibody AP33 (0.22 µg.mL^{-1}) in PBS in duplicate overnight at 4^{o}C to perform ELISA (Figure 3-2). After 16 hours, wells were incubated with 200 µL blocking buffer containing 5% milk w/v (PBST-M) for 2-4 h at room temperature. Plates were washed three times with 200 µL of wash buffer (PBST with 0.05% (v/v) Tween 20) to remove excess antibody. After washing, 50 µL of cell lysates containing HCV glycoproteins E1/E2 (H77) diluted 1/5 in PBST was added to each well and incubated for 2 h at room temperature. Subsequently wells were incubated with 50 µL of ficolins from different species diluted in a two-fold serial dilution (1/10 to 1/160) overnight at 4^{o}C. Washing was repeated and 50 µL of monoclonal anti-His-tag antibody (5 µg.mL^{-1}) in the wash buffer was added and incubated for 2 h at room temperature. After washing, the plate was incubated with 50 µL of alkaline phosphatase conjugated monoclonal anti-mouse IgG (Sigma) diluted 1/1000 in the wash buffer. Following another wash step, wells were incubated with 50 µL p-nitrophenyl phosphate (pNPP) substrate (SigmaFAST, Sigma) for 30 minutes to 1 h at 37^{o}C and the absorbance at 405 nm was measured.

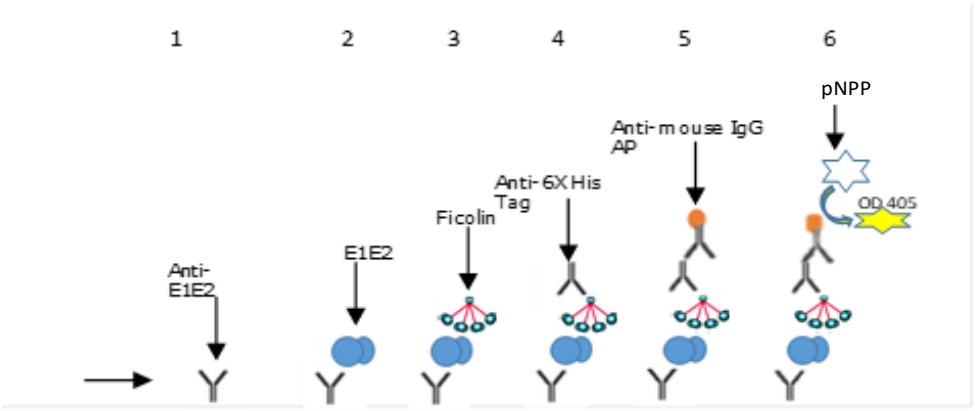

Figure 3-2 Ficolins and HCV glycoproteins binding assay (ELISA)

A schematic diagram showing the components used in ELISA to establish the binding of ficolins expressed from different mammalian species to HCV glycoproteins (E1/E2).

3.2.13 Statistical analysis

Experimental data were calculated as the mean value of repeated experiments, and the error bars represent the standard error of the mean (SEM) of each experiment. The statistical analyses were performed using One-way ANOVA with GraphPad Prism software version 7.

3.3 **Results**

3.3.1 Initial expression and purification of mouse ficolin

Recombinant FCNA and FCNB proteins were expressed in HEK 293T cells by transient transfection of cloned 6X His-tagged mouse ficolin expression constructs. Supernatants containing ficolin preparations were harvested and purified by nickel affinity chromatography. Fractions of 1 mL (eluates) were collected, and the purified recombinant mouse ficolin were studied by SDS – PAGE and immunoblot analysis (Figure 3-3). Only small amounts of FCNA or FCNB protein were observed in the load (L) or flow through (F) (Figure 3-3)**,** showing the affinity purification of protein. The fractioned eluates showed different banding patterns of these ficolins on western blot analysis. Proteins at 35 kDa, and above 180 kDa were observed using anti-polyhistidine tag monoclonal antibody then anti-rabbit IgG-HRP antibody as secondary antibody under non-reducing conditions (Figure 3-3 A), representing mouse ficolin monomers and multimers, respectively. Under reducing conditions (Figure 3-3 B) treated with DTT, these oligomeric forms were replaced by a monomeric form of a 33-35 kDa (FCNB) and 38 kDa (FCNA). However, there was BSA contamination observed at 60.5 kDa. Peak fractions were pooled, filtered and concentrated to 500 µL via ultrafiltration, followed by micro dialysis into 600 µL PBS to allow use in further analysis.

Western blotting was repeated using an alternative anti-His-tag antibody (4D11). This blot confirmed the ficolin protein but did not detect BSA contamination (data not

shown). The concentration of the purified mouse ficolin was determined using a BCA assay. A negative control protein preparation was generated by performing transfections in the absence of a ficolin-encoding plasmid. This was purified as the over-expressed FCNA/FCNB and used as a control preparation in the western blot. The purification, western blots and BCA assays showed that the negative sample contained minimal protein. This result implies that more optimization is required to ensure that negative control contains no protein.

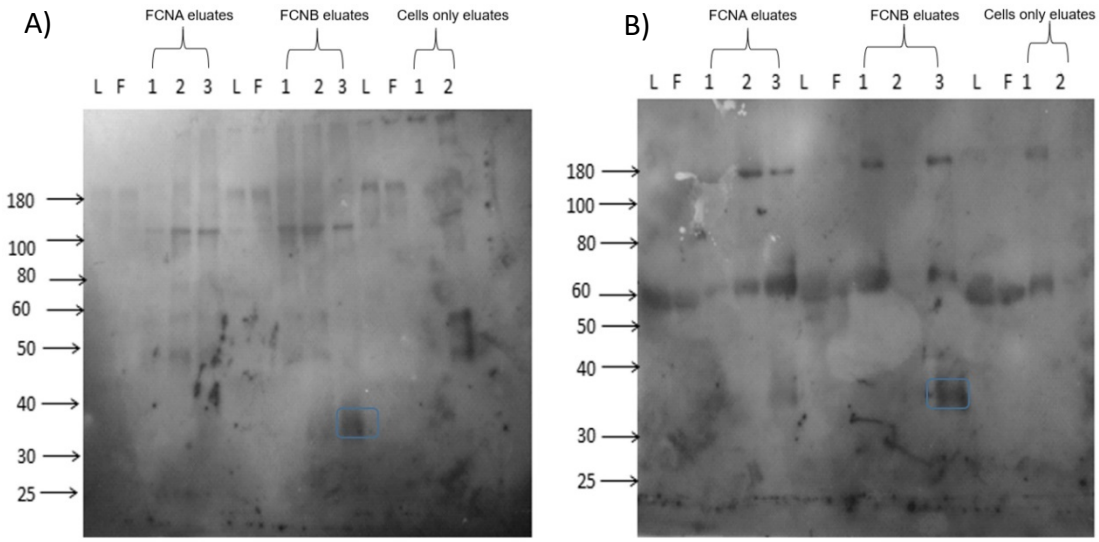

Figure 3-3 Analysis of the purified mouse ficolins using western blot

Transient expression of FCNA and FCNB genes in HEK 293T cells where L is load and F is flow-through. Harvested supernatants were purified with an AKTA purifier and analysed by western blot as shown. Proteins were detected using specific antibody a) non-reducing condition which showed a band at 35 kDa (monomers) for FCNB, 105 kDa (trimers) and above 180 kDa (multimers) for both proteins showing oligomeric composition. b) Under reducing conditions, the oligomers of FCNA and FCNB were reduced and a more prominent single band was detected at either 34 kDa, or 35 kDa for FCNA and FCNB, respectively.

To optimise the yield, protein expression was scaled up using increased numbers of seeded cells in larger culture flasks (T225 or T175). Ficolin-containing supernatants were harvested and concentrated to 1 mL by ultrafiltration and was not purified. The concentrated recombinant mouse ficolin was studied by SDS–PAGE and immunoblot. Under non-reducing conditions, proteins at 33-35 kDa, 37 kDa or above 100 kDa, were observed using anti-6X His-tag antibody (mouse monoclonal antibody), which did not have non-specific binding/background compared to the rabbit antibody that

was used previously. A polyclonal anti-mouse HRP antibody was used as a secondary antibody under non-reducing conditions (Figure 3-4 A) representing ficolin monomers and multimers**.** Under reducing conditions treated with DTT to a final concentration of 20 mM, these oligomeric forms were replaced by monomeric forms of molecular mass 33-35 kDa (FCNB), 38 kDa (FCNA) and 35kDa (human ficolin-2). The negative sample produced a faint band above 140 kDa under non-reduced condition (Figure 3-4 A) but no clear band was visible under reduced condition (Figure 3-4 B). The concentration of the unpurified protein preparation was determined, using a BCA assay; 4658 µg/mL (FCNA), 3682 $\mu g.mL^{-1}$ (FCNB*),* 4875 $\mu g.mL^{-1}$ HumFCN2 and 2666 $\mu g.mL^{-1}$ (FCNNeg). The concentrated protein preparation without purification (unpurified) yielded better detection of the protein. This result implies that the increased number of cells and plasmids used during transfection yielded a better expression of mouse ficolins in HEK 293T cells. This initial optimisation of unpurified mouse ficolin was used as standard protocol to enable the expression of all the ficolins in this study. Hence, the conditions used for protein expression of unpurified proteins were consistent between different experimental protocols, of which two representative western blot experiments are shown below.

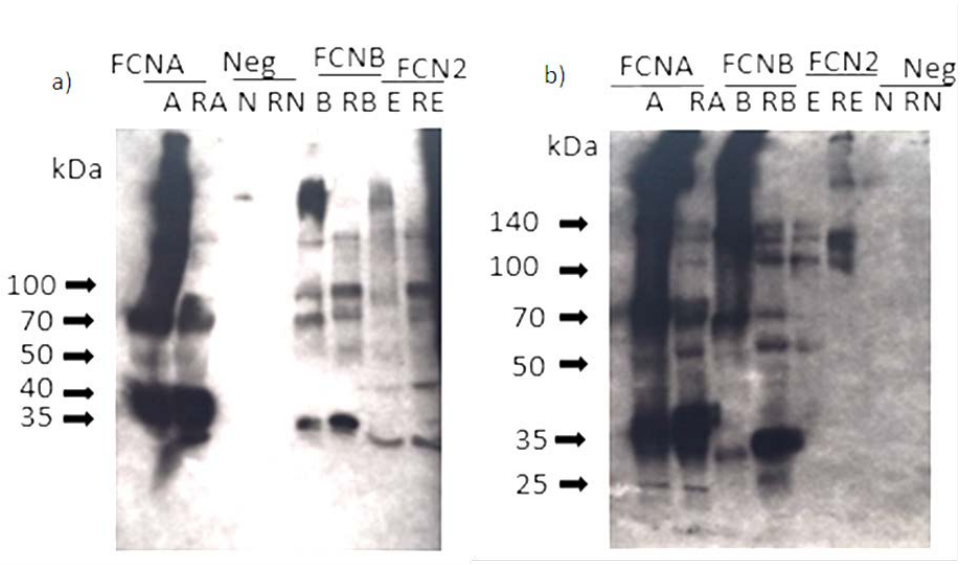

Figure 3-4 Analysis of the unpurified concentrated mouse ficolins using western blot

Transient expression of FCNA and FCNB gene in HEK 293T. Supernatants were harvested and concentrated by ultrafiltration. Proteins were detected using specific antibody (4D11). a) and b) are concentrated protein preparation of two different mouse and human ficolins at reducing and non- reducing conditions, where R is the reduced condition of all proteins RA (FCNA), RB (FCNB), RE HumFCN2 (E4) and N (Negative control) with same transfection condition. The single letters (A, B, E and N) represent non-reduced conditions. Mouse ficolins A and B showed bands at 33-35 kDa and 38 kDa monomers respectively, with their dimers and trimers showing oligomeric composition. More discrete bands were shown at reducing conditions.

3.3.2 Recombinant expression of all mammalian ficolins in HEK 293T cells

A similar procedure used to optimise yield as described above was employed. Proteins at 33-35 kDa, 38 kDa and above 250 kDa were observed using anti-His-tag antibody (Figure 3-5) representing ficolin monomers and multimers**.** Under reducing conditions, samples were treated with DTT to a final concentration of 20 mM, these oligomeric forms were replaced by monomeric forms; 33-35 kDa (FCNB), 38 kDa (FCNA), 34 kDa (gorilla ficolin-2 (GoFCN2)) and 35 kDa (human ficolin-2 (HumFCN2) molecular mass. The negative sample showed no evidence of protein**.** Although the GoFCN2 monomer did not appear as discrete as other ficolins, the oligomer showed that a potential functional higher-molecular-weight protein was produced (Figure 3-5). This result implies that the increase in the number of cells and plasmid used during

transfection yielded a better expression of ficolins in HEK 293T cells than using 10 cm dishes (data not shown).

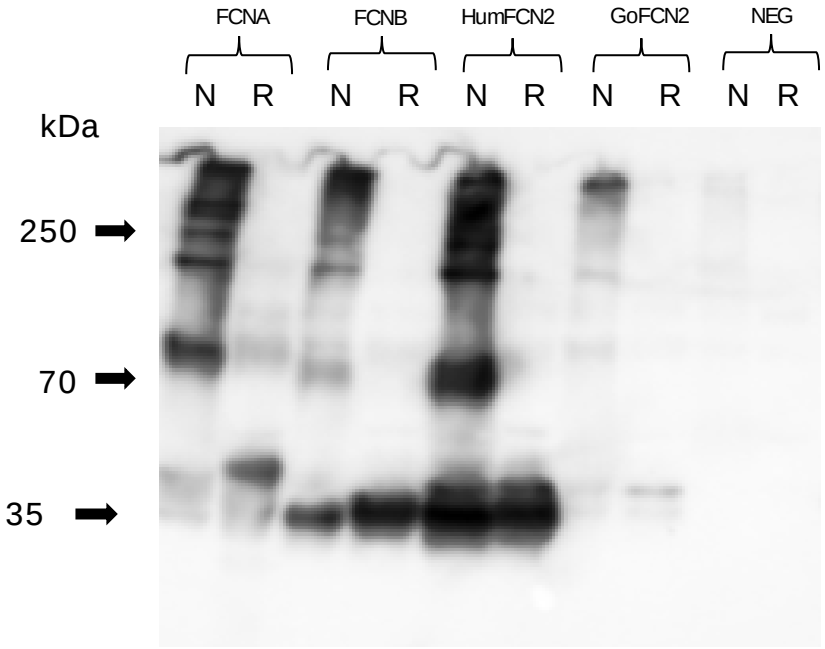

Figure 3-5: Expression of mouse, human and gorilla ficolins by transient transfection:

Transient expression of ficolin gene from different mammalian species in HEK 293T cells. Supernatants were harvested and concentrated by ultrafiltration. Proteins were detected using an anti-His tagged antibody. This shows concentrated proteins expressed by different mammalian species at non-reducing (NR) and reducing (R) conditions; mouse ficolin A (FCNA), mouse ficolin B (FCNB), gorilla ficolin-2 (GoFCN2), human ficolin-2 (HumFCN2) and NEG (Negative control) with same transfection condition. FCNA, FCNB, HumFCN2, GoFCN2 showed bands at 38 kDa, 33-35 kDa, 35 kDa and 34 kDa monomers respectively with their dimers and trimers showing oligomeric composition. More discrete bands were shown at reducing conditions. Indicated on the left are the molecular weights.

3.3.3 Calcium requirement of recombinant ficolins binding

Binding of all ficolins is mediated by S1 binding site and requires calcium for interaction in some instances [12, 38, 90]. To test if the binding of ficolins to AcBSA requires calcium ions, an ELISA was performed. Expressed recombinant ficolins were incubated with different concentrations of calcium and a calcium inhibitor (EDTA). Binding of unpurified ficolins in the presence of 0 mM, 0.5 mM, 5 mM and 50 mM of $CaCl_2$ was investigated (Figure 3-6). As a reference, the ficolin samples were diluted in TBST without calcium. The binding of all ficolins tested varied, with no significant differences in binding observed between the diverse concentrations (Figure 3-6 A-D).

An exception was FCNA (Figure 3-6 A), where there was lower absorbance showing lower binding at 50 mM, similar to the signal achieved with the negative control preparation. The negative control (Figure 3-6 E) gave lower signal in this assay than each of preparations of primate/murine ficolins. The absence of calcium (0 mM) had an effect on the binding of all the ficolins (Figure 3-6), suggesting a calcium-dependent interaction with AcBSA. Interestingly, the background signal achieved with a control cell supernatant was also dependent on the presence of $CaCl_2$, with higher ELISA signal observed in those reactions containing additional calcium.

The calcium-dependent nature of the interaction was also revealed by using a chelating agent. The data in Figure 3-7 show that the binding of recombinant ficolins to AcBSA was inhibited when EDTA was present, even at low concentrations. Each ficolin (Figure 3-7 A-E) bound to AcBSA in the absence of EDTA (0 mM) showing calcium dependent binding. This is likely to be caused by the interactions between the S1 binding site and AcBSA. This finding is consistent with an unpublished data in Dr Christopher Mason's PhD thesis where human ficolin-2 showed a binding affinity to AcBSA in the absence of EDTA (0 mM). Although the same binding was observed in the absence of calcium, the previously performed assays showed partial calcium-dependent binding. Once again, the negative control protein preparation demonstrated some binding signal in the absence of EDTA. This too was diminished when EDTA was added, indicating that supernatants of cells with a mock transfection produce a AcBSA-binding protein that cross-reacts with the anti-His-tag antibody utilised in these assays.

3.3.4 Binding specificity of recombinant ficolins to AcBSA

To test further whether the recombinant ficolins binding to AcBSA is acetyl-specific, AcBSA and BSA as negative control were coated onto plates and analysed by ELISA (Figure 3-8). Mouse and human ficolins were added in a two-fold serial dilution, and their binding was determined. The assay was performed in a 5 mM calcium-containing

buffer. This result confirmed that all the recombinant ficolins expressed could bind to AcBSA in a dose-dependent manner, and no binding to BSA was observed (see Figure 3-8). This result implies that ficolins expressed in this study as also observed for other human ficolins [25] show preference for acetylated compounds.

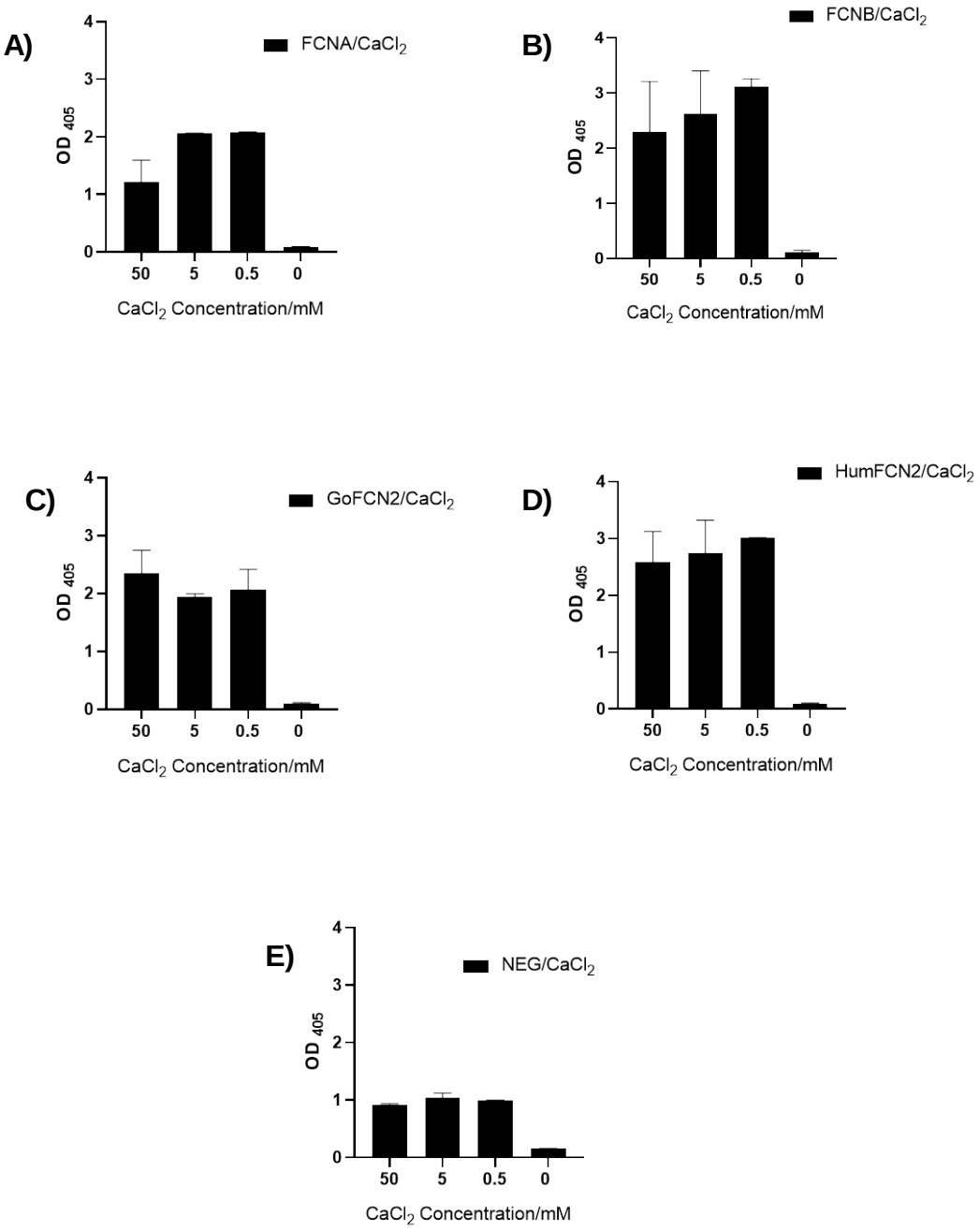

Figure 3-6: The effect of calcium on the binding of unpurified recombinant ficolins preparations

Different mammalian recombinant unpurified ficolin binding to AcBSA-coated microtiter wells in the presence of varying concentrations of $CaCl_2$; 50 mM, 0.5 mM, 5 mM and 0 mM. A) FCNA B) FCNB C) GoFCN2 D) HumFCN2 E) Negative sample (NEG). This assay was performed in the absence of calcium in the binding/dilution buffer. No binding of ficolin to AcBSA was observed in the absence of calcium ions, implying a calcium-dependent assay, although the different concentrations of calcium did not seem to make any difference. Bound ficolins were detected with an anti-His-tag antibody. Results shown is a representative of three independent assays. Error bars represent the standard error of mean.

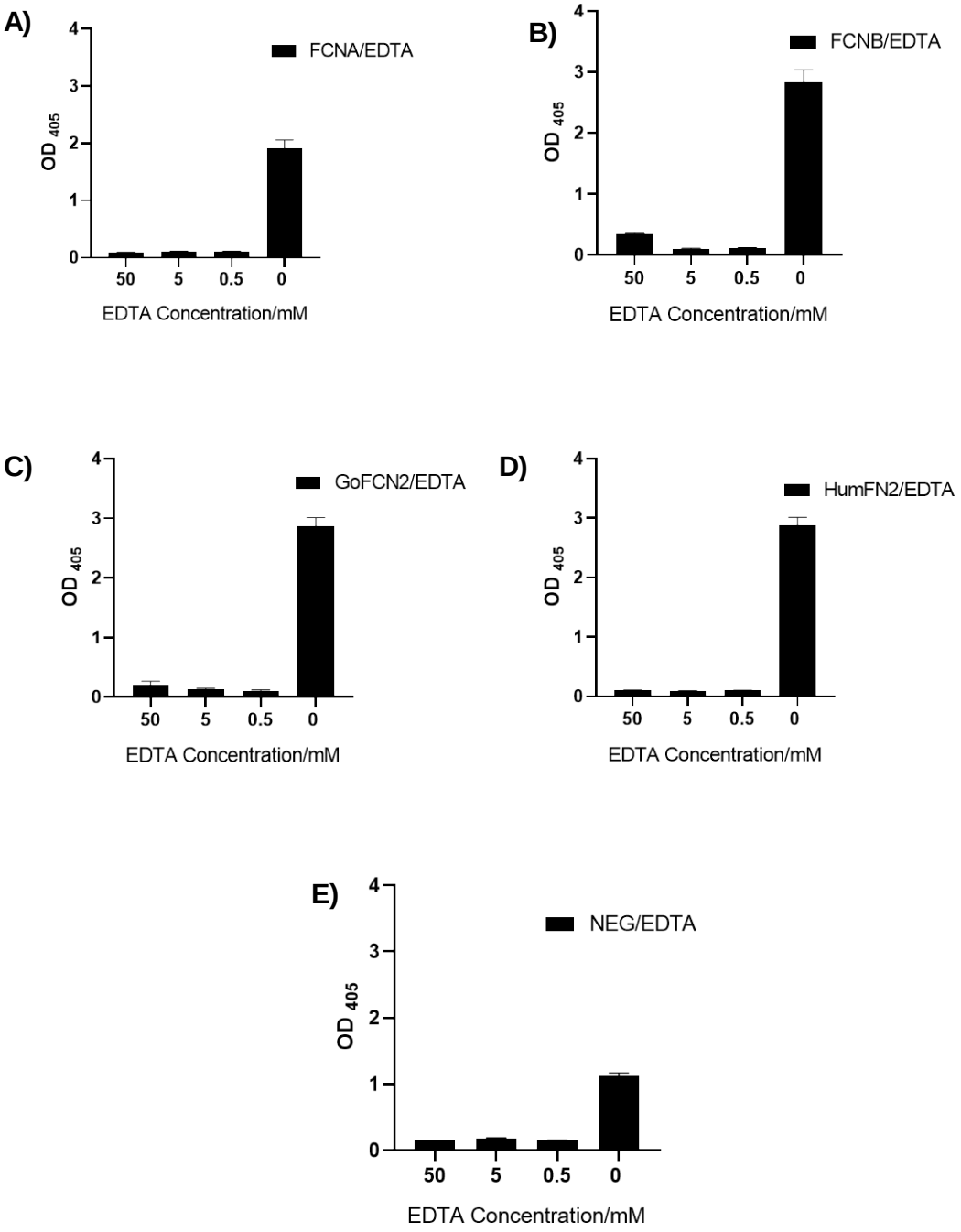

Figure 3-7: The effect of EDTA on binding of unpurified recombinant ficolin preparations to acetylated BSA

Different mammalian recombinant unpurified ficolin binding to AcBSA- coated microtiter wells in the presence of varying concentrations of EDTA; 50 mM, 0.5 mM, 5 mM and 0 mM. (A) FCNA B) FCNB C) GoFCN2 D) HumFCN2 E) NEG (supernatant from mock-transfected cells). This assay was performed in the presence of calcium in the binding/dilution buffer. EDTA inhibited the binding of all unpurified ficolins to AcBSA at different concentrations. The binding of all ficolins to AcBSA in the absence of EDTA shows a calcium-dependent binding. Error bars represent the standard error of mean.

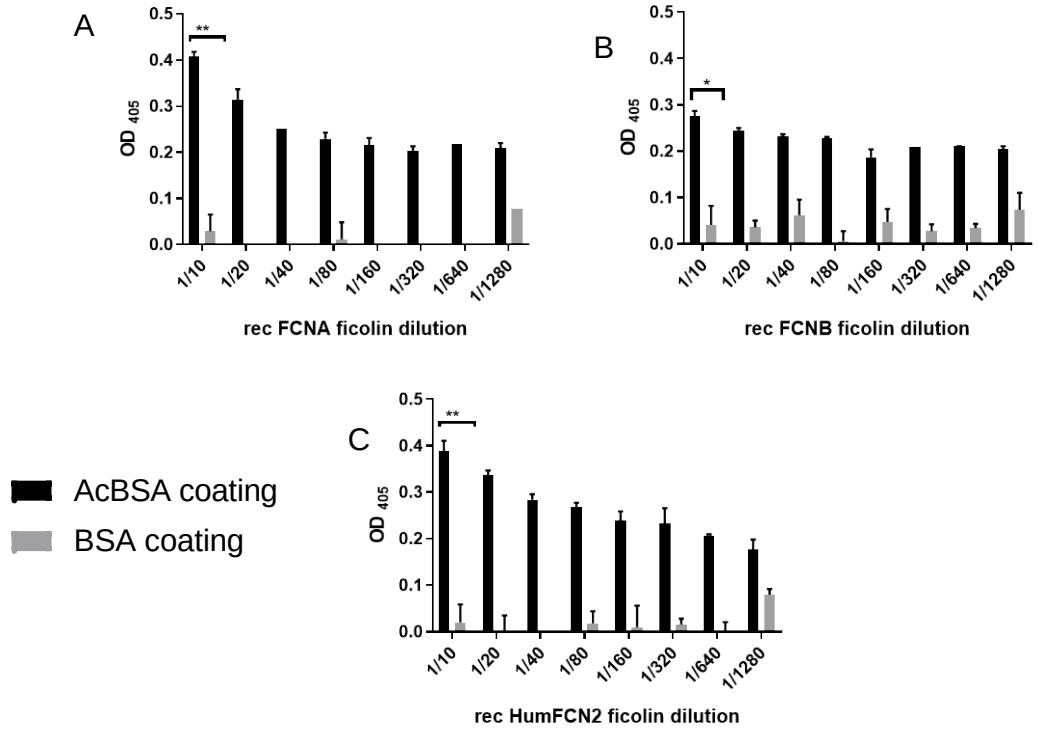

Figure 3-8 Binding of unpurified recombinant ficolin preparations to AcBSA and BSA

Mouse and human ficolin-2 binding to either AcBSA or BSA coated plates were compared by ELISA at a 2-fold dilution. A) FCNA B) FCNB and C) human ficolin-2. This assay was carried out in the presence of 5 mM calcium chloride in the dilution/binding buffer. All ficolins bound to AcBSA in a dose-dependent manner, with no effect on BSA. Using an unpaired t-test each FCNB binding to AcBSA compared to BSA was found to be significant with a value of p< 0.05 (*) while FCNA and human ficolin-2 was found to be significant at the value of p < 0.001 were indicated with **.

3.3.5 Purification of recombinant ficolins expressed in HEK 293Tcells

3.3.5.1 Purification by Immobilized metal affinity chromatography

Supernatants containing His-tagged ficolins were firstly purified by metal affinity chromatography, using 20 mM to 50 mM imidazole for binding of the ficolin and a stepwise gradient to elute bound proteins. Fractions were collected and analysed on SDS-PAGE and immunoblot analysis. Purification of each ficolin preparation (Figure 3-9) illustrated that both human ficolin-2 and mouse ficolin A were detected, however, there was no evidence of bands on the gel for the sample containing gorilla ficolin-2. For the human protein, ficolin-2 was observed at 35 kDa, 70 kDa and 250 kDa,

representing monomers, dimers and higher oligomers, respectively. None of the fractionated eluates from the sample transfected with gorilla ficolin-2 contained ficolin-2 (Figure 3-9 B). Mouse ficolin A revealed monomers in eluates 26 and 30, with a prominent band in fraction 28 and faint higher oligomers. Although no ficolin was observed in flow-through when probed with an anti-His-tag antibody, ficolins and other cellular proteins were detected in flow-through when silver stained (data not shown). This showed that some protein had passed through the column without binding.

3.3.5.1 Improvement of ficolin purification method

Further optimisation of the purification method was required due to ficolin not completely binding to the column and no absorbance peak being detected by spectrophotometry. Also, purification by a column-based method could take several hours; hence an alternative, more rapid method was used (MagneHis protein purification). Supernatants from 4 x 175 cm^2 culture flasks were concentrated and purified with MagneHis™ Ni particles. The purified fractions were analysed by using the anti-His antibody for detection of ficolins by western blot (Figure 3-10). To increase the purity and ensure separation of monomers and oligomers, harvested ficolin were concentrated to 2 mL (Figure 3-10 A) and 5 mL (Figure 3-10 B). The purified fraction of the 2 mL concentrated ficolin showed faint bands under non-reducing conditions (Figure 3-10, lane 1). Under reducing conditions (Figure 3-10 A, lane 2), the oligomers were reduced to monomers (38 kDa for FCNA and 35 kDa for human ficolin-2) by adding 2-mercaptoethanol. For the 5 mL concentrated ficolin, the starting material (S) of the western blot for both FCNA and human ficolin-2 shows monomeric and oligomeric forms of the proteins prior to purification (Figure 3-10 B). Lanes 1 and 2 showed that the fractions contain monomeric (35-38 kDa), dimeric (70-74 kDa) and multimeric proteins (250 kDa and above). For the purified fractions of FCNA, a notable band was observed above 250 kDa corresponding to a 12-mer oligomer, which can be predicted to comprise four ficolin trimers. Such oligomer assembly has also been exhibited by human ficolin-2 [16].

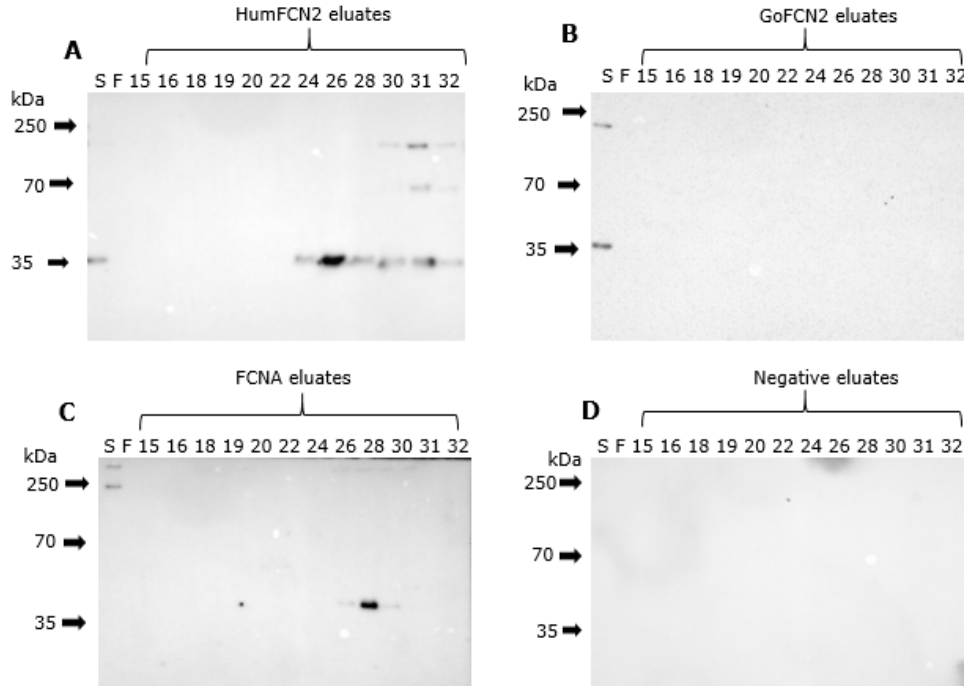

Figure 3-9 Purification of His-tagged ficolins by IMAC

Supernatants were harvested, purified using nickel agarose affinity chromatography, and eluted fractions were subjected to SDS-PAGE and immunoblot analysis. The start material (S), flow through (F) and eluted fractions were electrophoresed in 10% gels. The blots were developed using anti-His tag monoclonal antibody (His.H8) and anti-mouse polyclonal horseradish peroxidase (HRP) secondary antibody. A) human ficolin-2 (HumFCN2) is visible as a monomer of 35 kDa in fractions 24, 26 and 28. Oligomeric bands are represented in fractions 30-32 B) gorilla ficolin-2 (GoFCN2); no GoFCN2 was visible in the eluted fractions. C) Mouse ficolin A (FCNA); faint monomers and multimer bands were visible in fractions 26 and 30 with a more prominent monomer at 38 kDa detected in fraction 28. The molecular weight markers are shown on the left. D) Negative control with no bands was detected.

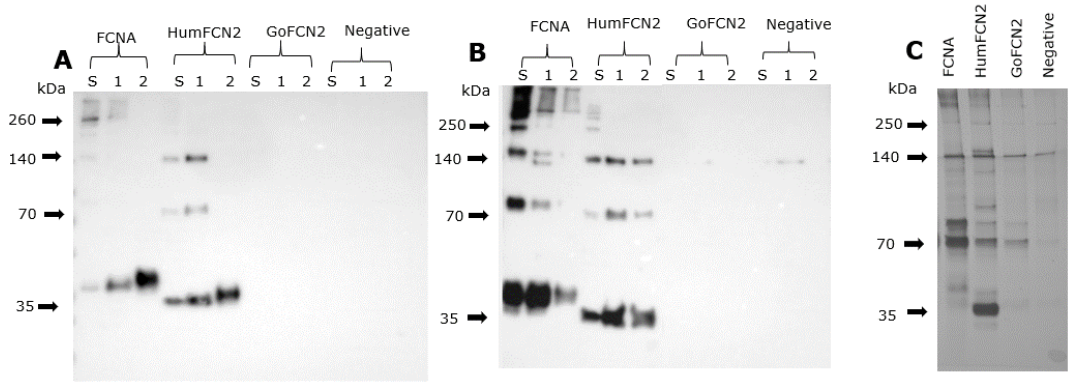

Figure 3-10: SDS-PAGE analysis of recombinant ficolins purified by Magnehis beads

Recombinant ficolins from different mammalian species were purified using a MagneHis kit. The proteins were eluted with 500 mM imidazole. Samples were analysed on 10% polyacrylamide gels followed by (A), (B) western blots and (C) silver stain of pooled purified fractions. The blots (A) and (B) were developed with anti-His tag (His. H8) monoclonal antibody. Indicated on the blots are S (starting material) with purified ficolins in lane 1 and 2. A) and B) are blots displaying samples concentrated to 2 mL and 5 mL with non-reducing and reducing condition in lane 1 and 2, respectively. Monomers, dimers and multimers were observed for mouse ficolin A (FCNA) and human ficolin-2 (HumFCN2), but not for gorilla ficolin-2 (GoFCN2) or the negative control. The eluates were pooled and analysed by silver staining (C) which revealed different proteins other than the mouse, human and gorilla ficolin monomers and multimers shown. No bands were visible in the negative control except at about 140 kDa. Indicated on the left are the estimated molecular weights based on the molecular weight standards.

3.3.6 Calcium requirement for binding of purified recombinant ficolins to acetylated BSA

An ELISA assay was performed to test the calcium requirement of the purified ficolins using different concentrations of calcium (Figure 3-11) and EDTA (Figure 3-12). There was little or no binding of purified FCNA, FCNB and HumFCN2 ficolins to AcBSA (Figure 3-11 A, B & D) when compared to negative control (NEG) (Figure 3-11 E), which had no ficolin as detected by western blot. While there was some evidence of binding of the gorilla ficolin-2 (GoFCN2) AcBSA at a calcium concentration of 5 mM, the error in this assay makes it very difficult to determine the validity of the data. These purified ficolin preparations were diluted in TBST without calcium. When the same assay was repeated for different concentrations of EDTA (Figure 3-12), the signal achieved in the AcBSA binding assay was reduced further. In this case the ficolin samples in the EDTA assay were diluted in TBST-Ca. No binding was observed in

the absence of EDTA (0 mM). Together, these results suggest that there was insufficient purified ficolin present in these preparations to facilitate detectable signal.

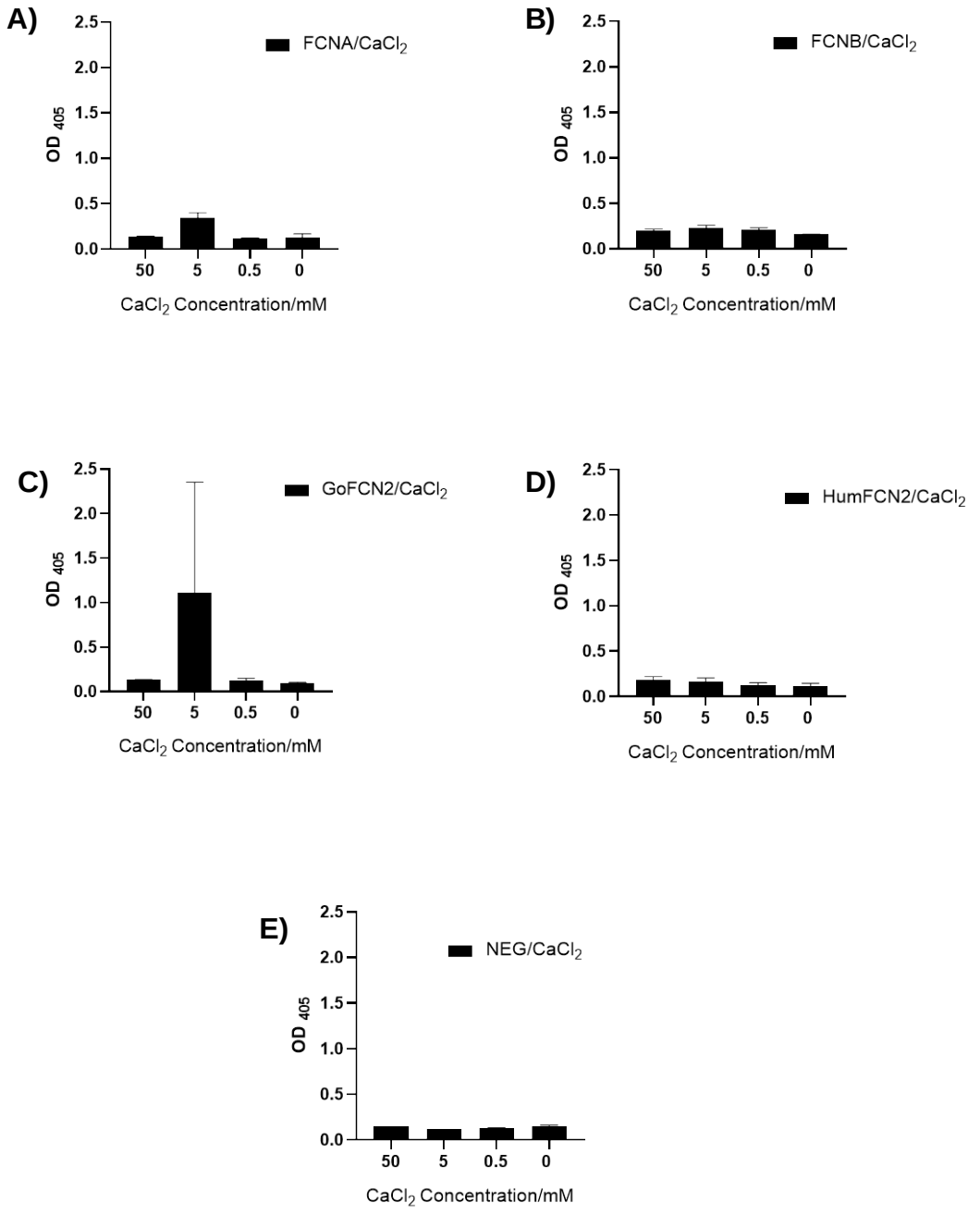

Figure 3-11 The effect of calcium on purified recombinant ficolin binding

Different mammalian recombinant purified ficolin binding to AcBSA-coated microtiter wells in the presence of varying concentrations of $CaCl_2$; 50 mM, 0.5 mM, 5 mM and 0 mM. (A) FCNA B) FCNB C) GoFCN2 D) HumFCN2 E) NEG sample. This assay was performed in the absence of calcium in the binding/dilution buffer. All ficolin except GoFCN2 (C) at 5 mM showed little or no binding to AcBSA. GoFCN2 bound to AcBSA at a calcium concentration of 5 mM. Error bars represent the standard error of mean.

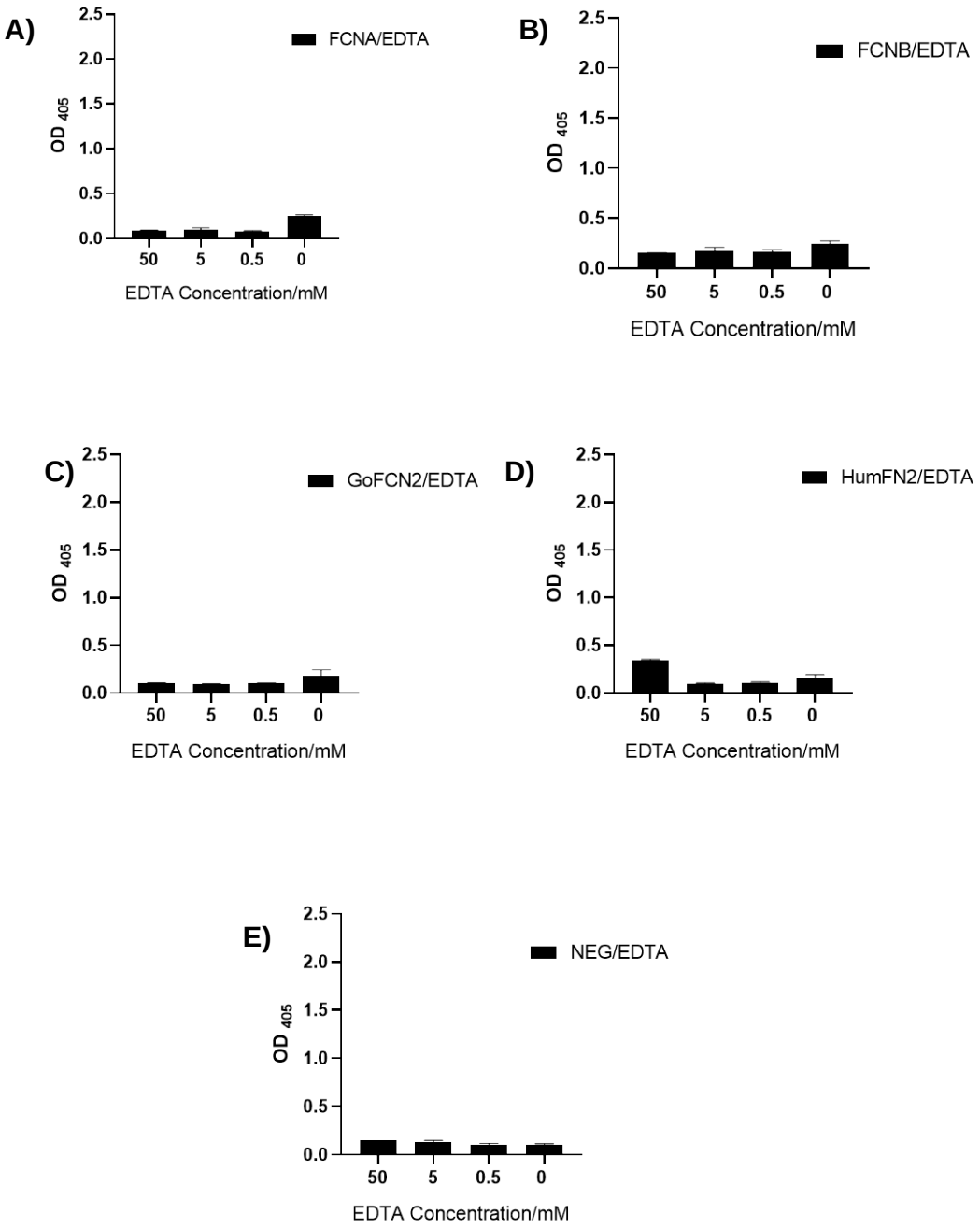

Figure 3-12: The effect of EDTA on purified recombinant ficolin binding

Different mammalian recombinant purified ficolin binding to AcBSA-coated microtiter wells in the presence of varying concentrations of EDTA; 50 mM, 0.5 mM, 5 mM and 0 mM. (A) FCNA B) FCNB C) GoFCN2 D) HumFCN2 E) NEG. This assay was performed in the presence of calcium in the binding/dilution buffer. There is no evidence of binding of all purified ficolins to AcBSA at different concentrations, even in the absence of EDTA. This shows there was little or no ficolin present for the EDTA to inhibit. Error bars represent the standard error of mean.

3.3.7 E1/E2 glycoproteins detected in cell lysates

The plasmid encoding the full-length H77 E1/E2 coding region (aa170-746) was expressed in HEK 293T cells to express the E1/E2 glycoproteins. The cell lysates were used as a source of E1/E2 to mimic the native forms present on the virus particles. Proteins were separated using non-reducing 10% SDS-PAGE by western blotting with the broadly reactive anti-E2 antibody ALP98 that targets linear epitopes [298]. Evidence of E2 monomers were observed at 70 kDa (Figure 3-13). Disulphide linked aggregates seen at higher molecular weight (105 kDa) may probably represent misfolded complexes [140]. Previously, a combination of CD81 pull down assay and antibody detection studies from our research group and other groups aimed at both linear and conformation epitopes have shown that the E1/E2 clone used in this work was correctly folded [290, 295, 296, 298, 299].

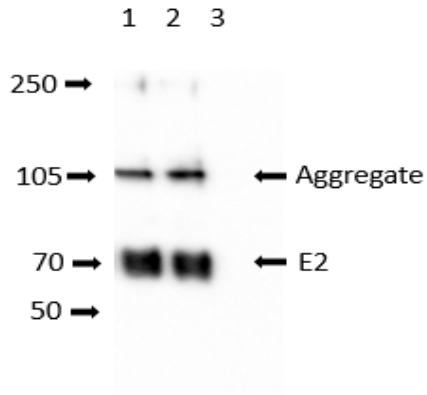

Figure 3-13 Detection of E1/E2 glycoproteins in HEK 293 T cells by western blotting

E1E2 constructs were expressed in HEK 293T cells and analysed by non-reducing western blotting. 7 µL of the protein was loaded on 10% gel. Monomeric E2 protein was detected using the broadly reactive anti-E2 monoclonal antibody (ALP98). The molecular weight of the protein in kDa is shown on the left-hand side. The blot showed bands at 70 kDa relating to E2 monomers (lane 1 and 2). Untransfected 293T cells lysate was used as negative control (lane 3). The blot was revealed by using the G-Box Syngene image system.

3.3.8 Recombinant ficolins expressed by different mammalian species bound to HCV glycoproteins

To assess whether recombinant ficolins expressed by different species can bind to HCV glycoproteins, an ELISA assay was employed with a fixed concentration of the target molecule and a dilution series of ficolins (Figure 3-14). Anti-E2 monoclonal antibody (mAb), AP33 was used to capture E1/E2 glycoproteins onto ELISA plates. Recombinant ficolins were added in a two-fold dilution series and incubation was followed by washing to remove unbound protein. The bound glycoproteins were revealed using an anti-His-tag monoclonal antibody as described in materials and methods (section 3.2.12.3). Ficolins from different species were shown to bind in a dose-dependent manner, this binding was calcium-dependent due to the calcium binding sites present in the FBG domain of each ficolin. Because binding was calcium dependent, 5 mM calcium was included in the wash buffer. Ficolin binding to the E1/E2 glycoproteins was dose dependent. In contrast to the AcBSA binding assays, negligible background binding was observed using a sample from a mock-transfected control.

3.4 **Discussion**

The innate immune system is the first line of defence against pathogens to eliminate them by triggering inflammatory responses [300]. It uses a variety of PRRs, including ficolin, which is an example of a defence collagen recognising PAMPs such as glycans on the surface of viruses. Binding of ficolins to pathogens leads to complement activation. Ficolin-coding genes have been identified in *Homo sapiens* and other species. It also contributes to the innate immunity of other viruses and bacteria [13, 37-40]. Hence human ficolin-2 is unique compared to other types of human ficolins as it has a broader recognition spectrum and also evidence of polymorphism because of single nucleotide polymorphisms [72, 301]. The well-studied characterisation of human ficolin-2 will enable a better understanding of other

ficolins. To date, there have been no published studies on the interaction of ficolins from non-human species and different viruses. This study provides the first evidence of mouse and gorilla ficolins to HCV glycoproteins.

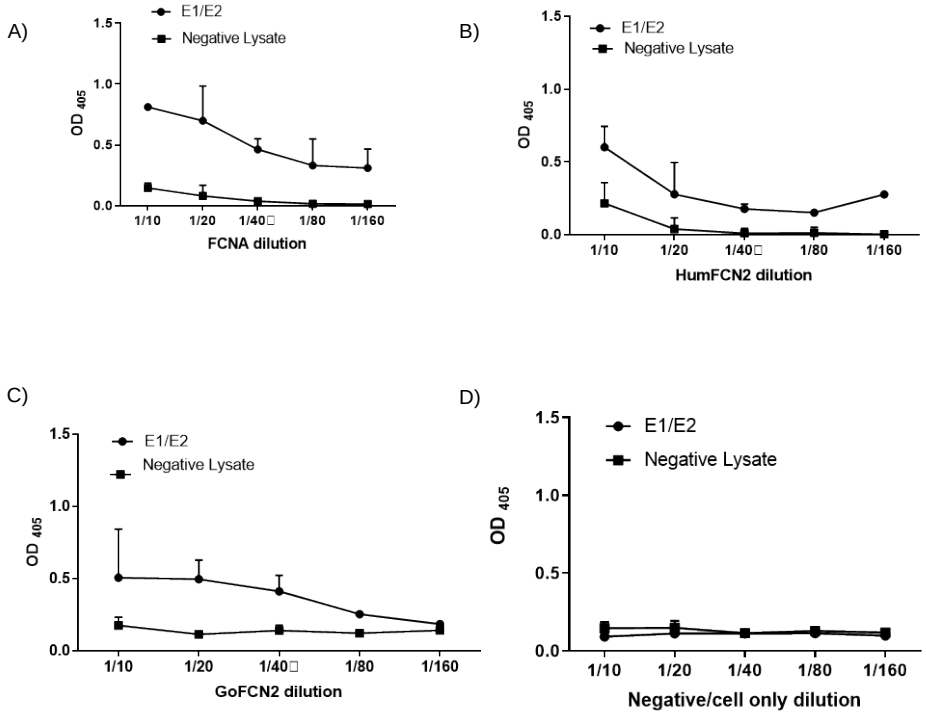

Figure 3-14 Binding of ficolins to HCV glycoproteins E1/E2

The ability of ficolins; a) mouse ficolin A (FCNA) b) human ficolin-2 (HumFCN2) and c) gorilla ficolin-2 (GoFCN2) to bind to HCV E1/E2 (●) was evaluated by ELISA. E1/E2 was captured by a monoclonal anti-E2 antibody, AP33 at 0.22 µg.mL^{-1} and a serial dilution of ficolins expressed from different mammalian species were added. Bound ficolins were detected by anti-His-tag antibody at OD 405. The binding of the concentration of E1/E2 in the cell lysate was directly related to the dilution of each ficolin. Negative control lysate (▮) was included. A negative control lysate from untransfected cells and negative control in which no ficolin was added (d) was used to show the specificity of the interaction.

To biochemically characterise the interaction between HCV and ficolins expressed by a range of diverse mammalian species, it was necessary to produce purified recombinant forms of these proteins. Successful expression of recombinant proteins depends on the properties of the protein and expression system. To produce pure ficolin from different mammalian species, free of contamination and other serum lectins, the recombinant proteins were produced in a mammalian expression system (HEK 293T cells), where the cells do not naturally express ficolin-2 [302]. This is

consistent with other studies [34, 303, 304] where other types of mammalian expression systems such as the Chinese Hamster ovary (CHO) cells [13] have been used to express recombinant proteins of different mammalian ficolins. For the first time, the coding region of NHP *FCN2* gene was cloned into pcDNA3.1 expression vector and a soluble His-tagged gorilla ficolin-2 was expressed in 293T cells. The protein was designed with an amino (N)-terminal 6X His-tag, allowing it to be easily detected and purified using an anti-His antibody or a more specific antibody (GN5 or FCN2-K19). The N-terminus was tagged since the C-terminal region (fibrinogen domain) is critical for binding of the protein to different ligands, and this might affect recognition if modified. The complexities of labelling the C-terminus of ficolin-2 have been previously demonstrated (Dr Christopher Mason-PhD thesis; unpublished data) to impact expression. Interestingly, the N-terminus of ficolin-2 is cysteine rich, more flexible, and is responsible for the oligomerization of the protein by disulphide bonding [280] and will not affect the binding specificity of the protein. Two cloned N-terminal His-tagged mouse ficolin expression constructs were purchased, transformed and expressed in HEK 293T cells. The expression plasmids were introduced into HEK 293T cells as reported earlier [305]. Evidence of ficolins expressed in cells were confirmed, albeit at low levels. To improve expression, the transfection was altered. Cells were seeded at 1 x 10^7 cells/mL in 225 cm^2 or 5 x 10^6 cells/mL in 175 cm^2 flasks, and an increase in protein expression was observed. This is consistent with a study where cells were seeded at 6 x 10^6 cells/mL in a T225 flask to improve the expression of recombinant soluble HIV envelope glycoproteins [306].

The functionality of the ficolins expressed by different mammalian species depends on the oligomerization of the recombinant proteins. Therefore, the oligomerization of recombinant mouse ficolins and gorilla ficolin-2 were compared to that of human ficolin-2 which has been previously studied [13, 90]. In this study, recombinant proteins expressed by different ficolin species showed a characteristic oligomeric pattern in consensus with previous discoveries [42, 307]. As expected, a mixture of

monomers and multimers were found at non-reducing conditions whereas discrete bands of all the proteins were prominent when the disulphide bond was reduced with a reducing agent (Figure 3-5). The mouse ficolins correspond to other FCNA and FCNB mouse ficolin proteins previously described; although the specificity of the antibodies used for detection were different [9, 16, 33, 308]. Even if the gorilla ficolin-2 expression was low in this study, the oligomeric profile shown (Figure 3-5, lane 7) can be compared to the 12-mers seen in the serum derived ficolin-2 from different non-human primates [11]. Hence the unpurified protein from the different species of ficolin appeared to form functional complexes.

The His-tag at the N-terminal enabled purification of the different recombinant expressed ficolins using Nickel agarose, as performed previously for the human ficolin-2 [13]. Although His-tagged ficolins were eluted using twice the imidazole concentration used in other studies [13, 90], the bound ficolin was not separated in all the samples, as shown in Figure 3-9 (A-D). In this study, the existing protocol (section 3.2.9.1) which requires many steps of processing and purification was not efficient, hence a more rapid process was used (section 3.2.9.2). This involved the use of paramagnetic pre-charged nickel particles (MagneHis™ Ni-Particles) to isolate polyhistidine protein directly from culture medium. Following MagneHis purification, the different recombinant ficolins also showed similar oligomeric pattern as the concentrated ficolins shown in the starting material (S) (Figure 3-10) from different species when visualised under non-reducing conditions by western blot. The starting material was re-purified to increase recovery after the first purification (Figure 3-10 B lane 2). Therefore, concentrating the protein facilitated the purification of the recombinant proteins cutting down a time-consuming process. Human ficolin-2 have been purified by other rapid and efficient methods using CELLine bioreactor [309] but this is the first time recombinant ficolins from different species have been purified using the MagneHis purification system. This suggests that more rapid methods are continuously being explored for the purification of ficolins. Consistent with the current

study, a previous study showed the use of spin concentrators to concentrate human ficolin-2 supernatants which were expressed in CHO cells [14]. This contradicts another study [309] where concentration and purification of the recombinant protein was not performed, because of precipitation and concentration of albumin that contaminated the purified human ficolin-2 preparation. The presence of a contaminant in ficolin samples produced in DMEM (10% FBS) was observed in this study after purification (Figure 3-3), hence the use of Opti-MEM (reduced serum media). Here we confirm that the use of a reduced serum medium (section 3.2.6) relieves contamination of albumin in DMEM whole media or having to concentrate albumin with supernatants, hence successful oligomerisation. This is consistent with other studies that used either serum-free media for ficolin expression or reduced serum media for other protein expression [25, 46, 310].

Comparing the unpurified and purified ficolin preparations, FCNA (Figure 3-5 and Figure 3-9 B) consistently showed doublets of the oligomers, probably caused by differences in glycosylation of two forms of the protein. This could be addressed in the future by digesting ficolin preparations with PNGase F, a glycoamidase with specificity for N-glycans [311, 312] to remove any possible glycans. Some proteins migrated at a clear size greater than predicted dodecamers, suggesting that ficolins expressed by species other than human can present with higher-molecular-weights. Together these results indicate that variations between the ficolins from different mammalian species do not interfere with the formation of disulphide interactions which are key to the formation of oligomers. In cysteine model studies, N-terminus contributes to the covalent structure of ficolin [313]. This is in line with the N-terminal domain disulphide bond formation of collectin multimerization [313-315]. A BCA assay was performed to determine the total concentration of protein present in these preparations; this assay could not determine how much ficolin was in each protein. No commercial antibodies against mouse and NHP ficolins are currently available to quantify the protein expressed in this study. The ELISA assay described in section

3.2.10.2 was used to test the binding specificities of the proteins based on AcBSA as a ligand molecule. This is consistent with another study where the interaction pattern of FCNA and human ficolin-2 were compared [316]. The fibrinogen domain enables each ficolin type to bind to different ligands, preferably N-acetylated compounds [317]**.** The interaction of ficolins to AcBSA confirms ficolin specificity for acetyl groups containing patterns as shown for human ficolin-2 in another study [38].

Binding of ficolins is mediated by binding sites in the C-terminal FBG domain, some of which require calcium for interaction [12]. This calcium dependent binding is due to the presence of calcium-binding sites found in the FBG domain [12, 35]. But there have been several controversial studies about this as reviewed in Chapter 1, section 1.4. Some studies have shown that, unlike collectins, human ficolin-2 could bind to GlcNAc in the absence of calcium [32, 42, 318] while other studies showed potential calcium binding sites within the FBG domain [48, 319]. Considering these controversies, this study investigated whether ficolins would bind to AcBSA in the presence or absence of calcium. In the unpurified ficolin preparations, ficolins were found to bind to AcBSA in a calcium-dependent manner (Figure 3-6) which was inhibited by EDTA (Figure 3-7). No ficolin binding was observed in the purified ficolin preparation (Figure 3-11 A, B, & D) (Figure 3-11 C). This binding was further reduced in the presence of EDTA (Figure 3-12). It is possible that the binding of AcBSA to GoFCN2 was calcium dependent, as binding was inhibited in the presence of EDTA (Figure 3-12 C). However, this is inconclusive as there was no reliable binding in the absence of EDTA. This inconsistency can to some extent be explained by the binding sites being indirectly affected by their ionic environments. The ionic environments can be varied by considering different pH conditions with/without calcium and addition of sodium chloride in the future. These conditions have been shown by other studies to affect the ficolin FBG domain conformational change, exposing the flexible C-terminus upon binding with different ligands [35, 38, 320]. In contrast to this study, it was shown by Dr Christopher Mason (PhD thesis; unpublished data) that the binding of human

ficolin-2 and its variants to AcBSA was partially calcium dependent as some calcium independence was shown in some variants in the presence of EDTA. Consistent with his findings is the unchanged effect of the different concentrations of calcium in the different species, although his study was focused on human ficolin-2 and its variants. Similar calcium-dependent binding was obtained with human ficolins, where binding to AcBSA was strongly inhibited in the presence of EDTA [14]. It could therefore be speculated that EDTA would inhibit/remove calcium ions, hence ficolins cannot interact with their ligands.

Structural insight into the recognition properties of non-human ficolins are yet to be determined, but the S1 binding site is said to be present in all ficolins as this represents their outer site [6, 12]. Likewise, the calcium ion binding site is present in a loop region that represents the most external part of ficolin trimers (Figure 1-3). Therefore, the interaction between AcBSA and the FBG domain of ficolins seen in this study may as well be organised and stabilised by the calcium ion. In addition, lectins including ficolins are said to have a small conformational change upon binding [41]. It thus seems possible that the disruption of this change when AcBSA binds to the FBG domain of ficolins might provide either calcium-dependent or independent binding. The specificity of this binding was examined by comparing it with BSA which is a non-acetylated compound in the presence of calcium (Figure 3-8). Ficolins interacted with AcBSA in the presence of 5 mM calcium in a dose-dependent manner, with no effect on BSA. This indicates that the ficolins from different mammalian species will bind differently to ligands, and this might seem similar for pathogens. As these results show binding of ficolins to AcBSA, it could be that this binding necessitates different requirements in the presence or absence of calcium depending on the interaction between ligands on the FBG domains.

HCV possesses two glycoproteins; E1 and E2 which are expressed as non-covalent E1/E2 heterodimers. They are glycosylated transmembrane proteins, containing 5 and 11 N-linked glycosylation sites respectively as reviewed in Chapter 1 section 1.11.4.1. As N-linked glycans might serve as ligands for mammalian ficolins, the potential ability of these ficolins to interact with HCV glycoproteins was investigated. Earlier studies have shown that HCV E1/E2 glycoproteins analysed by transient expression systems can form non-covalent heterodimers as well as heterogeneous disulphide aggregates [321, 322]. These noncovalent heterodimers are strongly suggested to be the prebudding form of the functional complex [139]. Extensive characterisation of full-length E1/E2 complex from different HCV genotypes expressed in mammalian cells have suggested that this form of HCV glycoproteins is fully functional [294, 295, 297, 323]. Additionally, analysis of the glycans bound to intracellular E1/E2 heterodimer have indicated that these are high-mannose type oligosaccharides possessing a $GlcNAc_2$ stem [117, 324] and are likely to be binding targets for ficolins. The HCV E1/E2 clone selected for this study had been shown previously to be correctly folded [295], interacted with human ficolin-2 [90] and evaluated for function using a retroviral pseudoparticle model of infectivity [225, 295, 325]. Here, the intracellular full-length E1/E2 (clone H77c) heterodimer was expressed in HEK 293T cells. Cells were lysed and intracellular HCV glycoproteins were detected with an anti-E2 mAb (ALP98) targeting a linear epitope (Figure 3-13). Albeit, the E1/E2 construct used in this study had been validated in our research group with conformation-dependent antibodies [295], this could be repeated in the future to confirm that the clone is in its native conformation for each assay to be performed. Results from an ELISA assay showed that HCV glycoproteins bound to ficolins from different mammalian species in a dose-dependent manner, see Figure 3-14. Similar to previous observations for human ficolins [25, 32, 38, 90], this study also shows that ficolins will bind to *N*-acetylated molecules, not non-acetylated molecules (Figure 3-8). The specificity of ficolins binding to their ligands can be emphasised by an inhibition assay (competition assays) which requires more investigation as this was not carried

out in this study. The ability of ficolin ligands to compete with the binding of different ficolins to a glycan or acetylated compound suggests that the binding is mediated by the ficolin FBG domain and ficolin specific ligand. The glycans on E1/E2 could be tested in the future by digesting with EndoH to support previous studies showing that the intracellular E1/E2 is made up of high-mannose type oligosaccharides.

Here, the binding activity of AcBSA and E1/E2 to ficolins was shown which proves that the expressed recombinant human, mouse and non-human primate ficolins are expressed as oligomerised multimeric forms. This part of the study faced some challenges which affected results. One of the biggest challenges was the lack of reference murine and non-human primate serum with quantified amounts of ficolin to produce standard curves for accurate quantification of each protein. Quantifying ficolins was also limited by the unavailability of specific antibodies for murine and primate ficolins. Use of His-tagged protein for which reference antibodies are available was an approach used to circumvent accurate quantification, permitting purified ficolins to be assayed for binding to viral glycoproteins. Purified preparations of ficolin were achieved and preliminary characterisation performed.

3.5 **Conclusions**

In this chapter the expression of recombinant proteins and preliminary analysis of ligand specificity were described. Expression varied between ficolins recovered from different mammalian species. The gorilla ficolin-2 demonstrated lower production yield compared to the mouse ficolins (FCNA and FCNB) and human ficolin-2. Interestingly, there was evidence of the oligomeric functional forms of all the ficolins expressed by different species, resulting in production of recombinant proteins that have the ability to bind various acetylated and glycosylated ligands. This is the first evidence of binding of non-human ficolin proteins to viral glycoproteins.

4 Production of Functional Retroviral Pseudotypes Bearing HCV and EqHV Glycoproteins for use in a Pseudotyped Based Neutralisation Assay

4.1 Aims

Although human ficolin-2 has been shown to interact with HCV glycoproteins, its spectrum of activity, and that of other ficolins from other mammalian species, is still poorly understood. It is unknown if ficolins bind to the glycoproteins of other hepaciviruses. This chapter aims to establish a model of entry for hepaciviruses other than HCV, using a retroviral pseudotype assay, with which to investigate the neutralizing activity of ficolins on different viral species. Hepacivirus pseudoparticles possessing E1 and E2 glycoproteins were produced to characterise their interaction with ficolins recovered from different mammalian species. The selection of retroviral packaging construct also influenced the function of hepacivirus pseudoparticles. Specifically, the infectivity of EqHV pseudoparticles was characterised. Infectious HCV and EqHV pseudoparticles possessing E1 and E2 glycoproteins were used to test the neutralising activity of the ficolins in a pseudoparticle infectivity assay.

4.2 Material and Methods

4.2.1 Cell lines and media

HEK 293T cells (MRC-University of Glasgow Centre for Virus Research) and Human hepatoma cells, Huh-7 [326] cultured at 37^{o}C in a humidified atmosphere with 5% CO_2 were used. Cells were grown in DMEM (GIBCO BRL, Paisley UK) supplemented with 10% FBS and 0.1 mM NEAA (complete medium). Stocks were passaged after approximately 80-90% confluence twice weekly after 72 h and maintained in a complete medium. HEK 293T-cells were seeded at a density of 1.2 x 10^6 cells/mL in Primaria 10 cm dishes. Subsequently, cells were transfected transiently using a reduced serum medium (Opti-MEM) (Gibco).

4.2.2 Plasmid constructs

Full-length clones of E1/E2 of HCV and EqHV were previously constructed by Dr Alexander Tarr. The plasmid encoding genotype 2a strain (J6) and genotype 1 isolate H77c (amino acid residues 170-746) E1/E2 glycoproteins were described previously [250, 327]. The EqHV variants (designated as 'PAA', 'PAAD', 'Thermo', 'Gibco' and 'Gibco 6.5') were provided by Professor Eike Steinmann, Ruhr University, Bochum, Germany.

pNL4-3.Luc.R–.E–, a pUC19 derived plasmid is the HIV core plasmid used in this study. This plasmid is a first-generation lentiviral construct which expresses the HIV *gag-pol* genes. The HIV accessory genes *vif, vpr, vpu* are defective in this construct with firefly luciferase gene inserted into the nef gene [252]. It was obtained from the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH.

A luciferase-encoding reporter plasmid (pTG126) and a murine leukaemia virus (MLV) *gag/pol*-encoding packaging construct (phCMV-5349) were gifts from Francois-Loic Cosset (Centre International de Recherche en Infectiologie, Ecole Normale Superieure de Lyon, France).

The cloned G glycoprotein of the vesicular stomatitis virus (VSV-G) was a kind donation from Didier Trono (plasmid pMD2G) (École Polytechnique Fédérale de Lausanne, Switzerland). Purified plasmids were prepared from TOP10 competent cells by transformation and purification using an HP Midiprep kit (sigma). The quality of the DNA was assessed by spectrophotometry at 260/280 nm using a Nanodrop (Thermo).

4.2.3 HCV and EqHV pseudoparticles (HCVpp and EqHVpp) Production

Full-length E1/E2 of HCV (H77 (genotype 1) and J6 (genotype 2a)) and EqHV (PAA, PAAD, Thermo, Gibco and Gibco 6.5) clones were generated based on previously described protocols [294, 328] and their amino acid sequences analysed. HCVpp and EqHVpp were produced as previously described [295]. They were produced by co-transfection with plasmids expressing the full-length E1/E2 glycoproteins (HCV and EqHV) and pNL4-3.Luc.R–.E– plasmid with a luciferase gene (two plasmid system) (Figure 4-1) or phCMV-MLV *gag/pol* packaging construct, a reporter plasmid containing an MLV UTR flanked with a luciferase gene and the full-length E1/E2 glycoproteins (HCV and EqHV) (three plasmid system) into the producer cell line, HEK 293T cells.

Briefly, HEK 293 T cells were seeded at a density of 1.2 x 10^6 cells/mL overnight prior to transfection in a 10 cm diameter Primaria dish (Corning) with 10 mL of complete DMEM with supplements as used in section 4.2.1. Transfections were performed with 2 µg of the three (MLV packaging vector and glycoprotein plasmid) or two plasmids (HIV packaging vector and glycoprotein plasmid) using 24 µL of PEI as gene delivering agent in 7 mL of Opti-MEM. Plasmids encoding HCV, EqHV, VSV and Ebola glycoproteins were transfected at 2 µg and 0.2 µg, respectively. After 4-6 h, the media was replaced with 10 mL complete DMEM. Subsequently, pseudoparticle-containing supernatants were harvested using a 10 mL syringe filtered through a 0.45 µm filter after 72 h. A negative control (∆E) generated in the absence of a glycoprotein plasmid was used for each transfection process. For purified pseudovirus production, supernatants were clarified of cell debris by centrifugation at low speed and 10 mL was gently loaded over a 20% sucrose cushion as described previously [329]. The virions were pelleted by ultracentrifugation in a Beckman SW40 ultracentrifuge for 2

h at 40,000 rpm (160 000 x g) and pellets were resuspended in 50 µL PBS for western blotting analysis.

4.2.4 Pseudoparticle infectivity assay

The infectivity of the pseudoparticles produced was tested using a human hepatoma cell line (Huh-7). Cells were seeded in triplicate in a 96 well white plate (Corning Inc.) at a density of 1.5 x 10^4 in 100 µL DMEM per well a day prior to infection. One hundred microlitres of the pseudoparticles were added to each well and incubated for 4 h and 150 µL complete DMEM was added. Following 72 h incubation, the media was discarded, and the cells were lysed by adding 50 µL of cell culture lysis reagent (Promega). The plates were kept on a rocking platform for 15 minutes until the cells were lysed. The luciferase reporter expression was quantified by luminescence using a FLUOstar Omega microplate reader (BMG Labtech). The pseudotype transduction was determined in relative light units (RLU). The plate reader was primed with luciferase substrate (Promega) which was dissolved in luciferase assay buffer (Promega). Fifty microlitres of luciferase substrate was injected, and the gain set at 3600 with the total light emission measured at 1 second integration time. The negative control (∆E) readings was used as the background noise in every assay. To avoid optic bleaching, the luminescence of the VSVpp was measured at a gain of 1800.

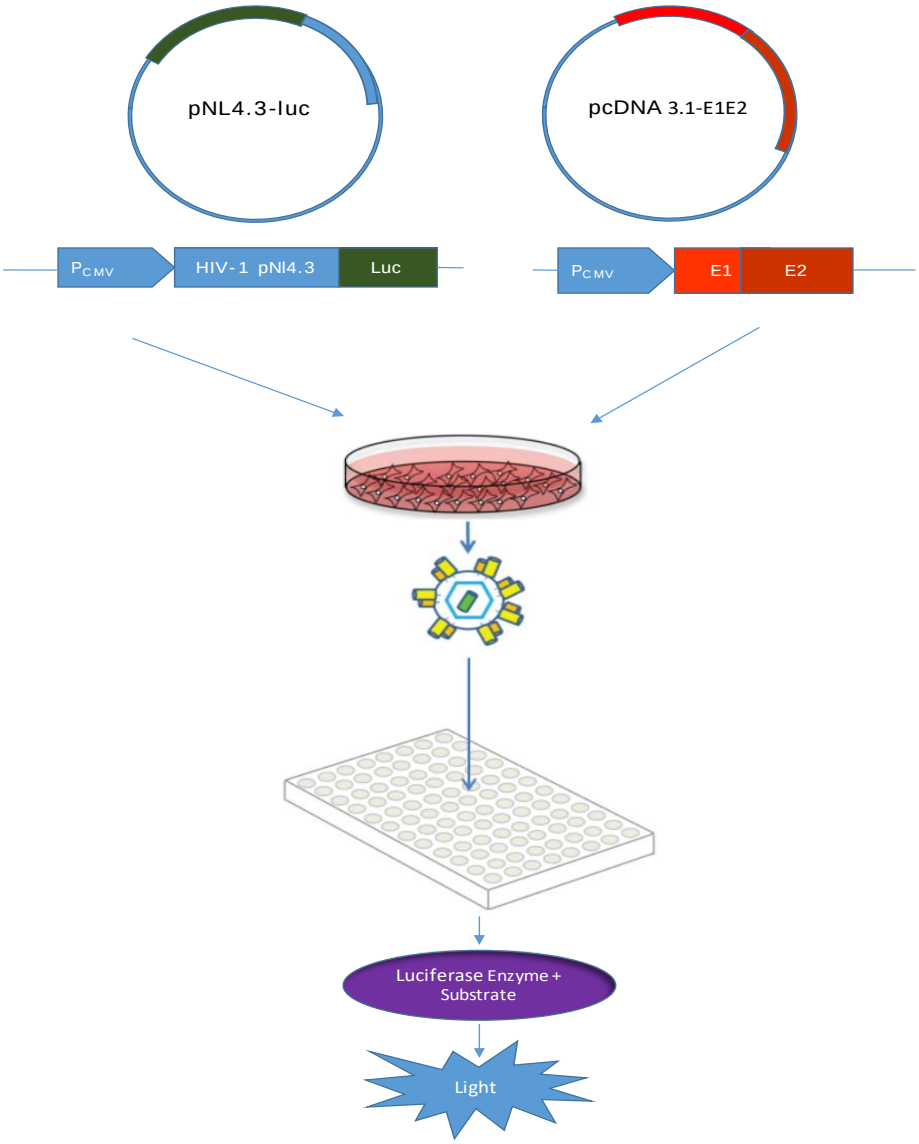

Figure 4-1 HIV based pseudotype production layout

The two-plasmid system shown was used for pseudotype production in this study. HIV-1 backbone plasmid (pNL4-3) carrying a reporter gene (luciferase) under the control of a CMV promoter was co-transfected with E1/E2-encoding plasmids (derived from pcDNA3.1) in HEK 293T cells. Huh-7 cells were infected in a 96 well plate with the pseudotypes generated. The infectivity was predicted by the luciferase activity in Huh-7 cells as a relative light unit (RLU).

4.2.5 Pseudoparticle neutralisation assay

Ficolin-mediated neutralisation was carried out similarly to an antibody neutralisation assay previously described [262]. Cells were seeded at the same density as the infection assay described above (section 4.2.4). In a V-bottomed 96 well microtiter plate (Sterilin), ficolins from different mammalian species were diluted in PBS in a 5-

fold serial dilution starting from 1/5 or 1/10 of the normalised protein (neat) of each ficolin to six other points (i.e. 1/5, 1/25, 1/125… or 1/10, 1/50, 1/250…) to have a final volume of 30 µL in each well. Subsequently, 270 µL of the pseudoparticles were added to each well to make a final volume of 300 µL and incubated for 1 h at room temperature. During the 1 h incubation, the media in the seeded plates was replaced with 50 µL of DMEM with supplements. After incubation, 90 µL of ficolin/pseudoparticle mixture was added to triplicate wells and incubated for 4 h at 37°C, 5% CO_2. Afterwards, 100 µL of DMEM was added. This was followed by a 72 h incubation after which cells were lysed by the addition of 50 µL of lysis buffer, mixing on a rocker for 15 minutes and the rest of the assay was completed as described in section 4.2.4.

4.2.6 Analysis

Luminescence were analysed using GraphPad Prism 6 software (GraphPad Software, Inc. San Diego, CA, USA). Infectivity was normalised to the luminescence of ΔE negative control (0%), which was glycoprotein deficient and uninhibited pseudoparticles control (100%) which was not preincubated with antibodies or ficolins. Hence the neutralising activity of antibodies and ficolins were expressed as percentage of uninhibited control. Results were represented as the standard error of mean.

4.3 **Results**

4.3.1 Evidence of Infectious hepacivirus pseudotypes expressing E1/E2 glycoproteins

HCVpp representing different HCV strains (genotype 1a (H77) and genotype 2a (J6)) and EqHVpp representing different variants (designated Thermo, PAA, PAAD, Gibco and Gibco 6.5) produced with HIV-derived backbone proteins were titrated unto Huh-7 target cells to assess their infectivity (Figure 4-2). Pseudoparticles possessing the

glycoproteins of either Ebola virus or VSV-G were used as positive controls, having been extensively optimised in our research group [247, 249]. Two negative controls were used in all the infectivity assays, a pseudotype virus bearing no envelope glycoprotein (ΔE) and a non-transduced cell-only control. Cells were incubated for 4 h, after which complete medium (DMEM) was added to the viral supernatants the infected cells were analysed for the expression of the luciferase reporter gene 72 h later. Both the HCV and EqHV (PAA) pseudotyped viruses infected the Huh-7 target cells with signals as high as 7.8 x 10^4 and 5.8 x 10^4 RLU, respectively (Figure 4-2).

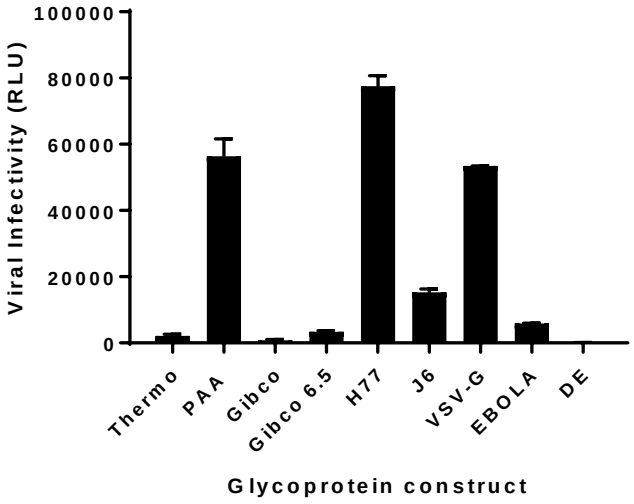

Figure 4-2 Infectivity of pseudoparticles possessing envelope glycoproteins isolated from different virus species

HIV core pseudotyped with E1/E2 glycoproteins of EqHV (Thermo, PAA, Gibco and Gibco 6.5), HCV (H77 and J6) and glycoproteins of VSV and Ebola. Pseudoparticles (PAA, H77, and VSV-G) efficiently infected Huh-7 cells while others show little or no infectivity. Infectivity of the pseudoparticles is measured by luciferase activity after 72 h. Error bars represent the standard error of the mean.

4.3.2 Influence of envelope plasmid expression on the production of EqHV pseudotype virus

It has been established that the ability to recover infectious HCVpp is influenced by the amount of encoding glycoprotein and the retrovirus-packaging construct plasmids delivered to the producer cells [247]. Using the routinely pseudotyped HCV as a model, the EqHVpp (PAA) possessing E1/E2 glycoproteins were produced with

varying amounts of E1/E2 plasmids and packaging vector for cell entry and neutralisation assays. To achieve this, a matrix of transfections was performed with serial dilutions of the EqHV (PAA) E1/E2-encoding plasmid. To determine the infectivity values for EqHV, pseudoparticles were first generated using varying amounts of pNL4.3 packaging construct and EqHV (PAA) E1/E2 glycoprotein construct (Figure 4-3). The maximal signal achieved for EqHV (PAA) infectivity was found to occur when using 1.6 µg of packaging construct plasmid and 1.6 µg glycoprotein-encoding plasmid during transfection. Reducing the amount of either plasmid during transfection had a dramatic effect on the infectivity achieved with EqHVpp (Figure 4-3 A). The amount of EqHV glycoprotein delivered to the transfected cells influenced the infectivity of the particles produced.

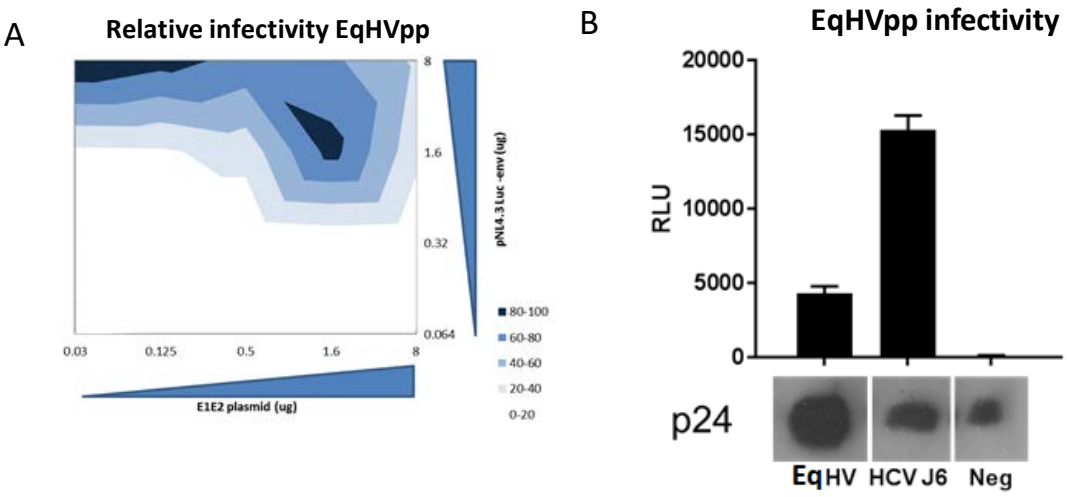

Figure 4-3 Plasmid quantity effect on EqHVpp infectivity and protein incorporation into pseudoparticles

Infectivity characterisation of EqHV (PAA) A) EqHVpp was made with a matrix of different quantities of the plasmids encoding the E1/E2 glycoproteins and the packaging vector. The graph is an x-y contour plot of pNL4.3 and E1/E2 encoding plasmid concentrations. The infectivity ranked with 20% increments is normalised to maximal infectivity, and the darker blue had the highest. B) Western blotting of pseudoparticles pelleted through 20% sucrose cushions with anti-p24 showing the detection of the capsid.

To determine if infectivity was linked with better incorporation of HIV capsid into EqHVpp and HCVpp, harvested viral particles from the supernatant of transfected

293T cells were purified by ultracentrifugation through sucrose cushions. Fractions were collected and analysed by western blot for p24 (the HIV-1 capsid protein) (Figure 4-3 B) using mouse anti-p24 mAb. The detection of p24 in the western blot correlated with the production of infectious particles, consistent with its incorporation into the particles.

4.3.3 HCV and EqHV entry are pH-dependent

During hepacivirus entry, the fusion event between viral and cellular membranes is triggered by low pH. This process is inhibited by different vacuolar acidification inhibitors [251]. To validate the entry of EqHVpp into Huh-7 cells, a vacuolar acidification inhibitor, bafilomycin A1 (BafA1) was used. Bafilomycin A1 inhibited cell entry of EqHVpp into Huh-7 cells and HCVpp, which served as control (Figure 4-4 A). Thus, entries of both EqHVpp and HCVpp into the cell are sensitive to bafilomycin A1. To investigate the entry pathway process in more detail, human and horse serum known to contain antibodies to both HCV and EqHV respectively were used in a neutralisation assay to assess their binding ability to EqHVpp and HCVpp (Figure 4-4 B). These results confirm the ability of each serum to inhibit pseudoparticle entry in a specific manner.

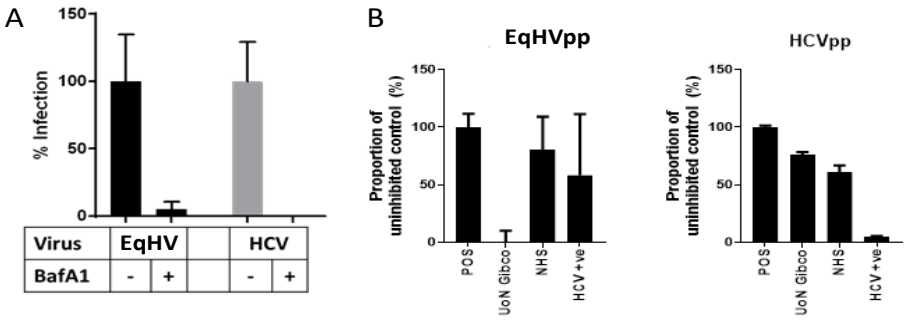

Figure 4-4 Validation of the entry pathway for EqHV

EqHVpp (PAA) were incubated with Huh-7 cells that had been previously treated with the inhibitor; Bafilomycin A1, or control containing standard media. B) Both EqHVpp and HCVpp preparations were treated with serum containing antibodies to either EqHV (UoN Gibco) or HCV (HCV+ve). Normal human serum (NHS) was used as a control for non-specific inhibition and compared with an uninhibited positive control (POS).

4.3.4 Production of HCV and EqHV pseudotype viruses with HIV and MLV cores/backbones

Having established that the quantity of glycoprotein and HIV packaging plasmids delivered to producer cells influenced the ability to recover infectious EqHVpp, the effect of species of retrovirus used as packaging construct was investigated. Several HCV and EqHV pseudotype supernatants were harvested; for both lentiviral (HIV) and gammaretroviral (MLV) pseudotypes. HCV strain J6 (genotype 2a) and EqHV variants were used to test whether they conferred any differences to the infectivity of the particles. Large differences in infectivity were observed in both HIV and MLV pseudotypes (Figure 4-5). Higher infectivity was observed for EqHV variants with HIV backbone systems compared to MLV backbones. While the EqHV E1/E2 variants gave little or no signal when using the MLV-based backbone, infectivity of the J6 (HCV E1/E2) with the MLV-signal was significantly high. The infectious titres of the HIV-based EqHVpp were generally found to be four to five-fold higher than those of the MLV-based EqHVpp (Figure 4-5), the HIV-based EqHVpp were used for subsequent experiments. Additionally, relative transduction titres of HCVpp and EqHVpp were higher than those of ΔE, showing that pseudoparticles bearing the envelope glycoprotein of HCV and EqHV were produced.

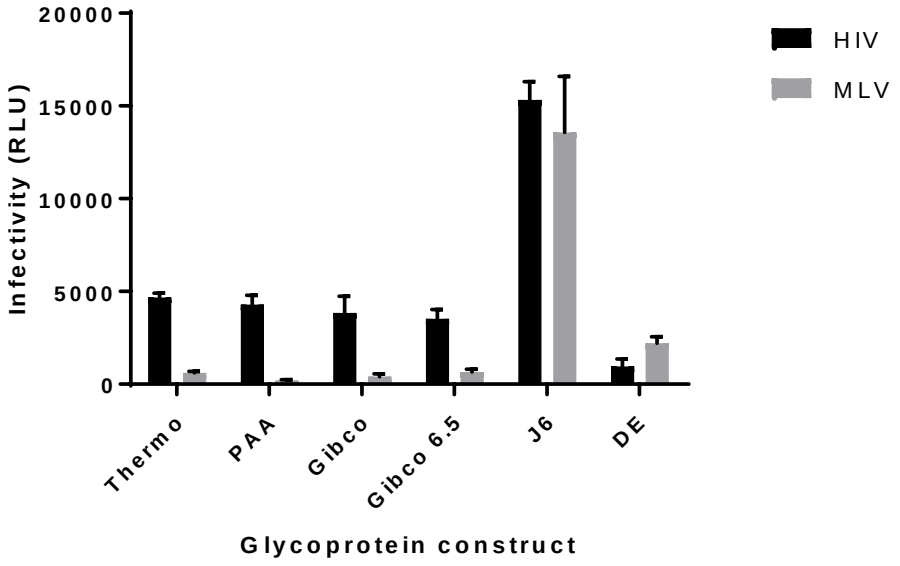

Figure 4-5 Infectivity of HCV and EqHV pseudotyped with HIV and MLV backbones

Pseudoparticles of four EqHV variants and HCV genotype 2a (J6) were produced with viral vectors derived from either HIV or MLV. Resulting pseudoparticles were infected with Huh-7 cells and luciferase activity was measured after 72 h of infection. All EqHV variants were infectious using HIV packaging vector but not infectious using an MLV packaging vector. Titres are displayed as RLU and the error bars are included as the standard error of the mean.

4.3.5 Pseudotype virus neutralisation assays

Having confirmed the infectivity phenotype of these particles and cell entry properties of E1/E2 displayed on HCVpp and EqHVpp, the neutralisation of the pseudoparticles were compared using different species' of ficolins in Huh-7 target cells. A two-fold serial dilution of *Galanthus nivalis* agglutinin (GNA), starting at a neat concentration of 10 µg.mL^{-1} to 0.3125 µg.mL^{-1} or five-fold from 200 µg.mL^{-1} to 0.064 µg.mL^{-1} or ficolins 1/10 to 1/31250, in triplicates, were preincubated with HCVpp, J6pp and EqHVpp, or Ebola and VSVGpp as a control for 1 h at room temperature. The EqHVpp showed E1/E2 glycoproteins from one EqHV variant; PAA while the HCVpp utilised E1/E2 glycoproteins from genotypes 1a (H77) and 2a (J6). The varying effects and abilities of GNA or ficolins (purified and unpurified) to inhibit infection of the target cells by different HIV-HCVpp and EqHVpp were investigated. The neutralising activity of

ficolins was expressed as the percentage of inhibition of pp infectivity compared to a control pp (uninhibited control) which was not incubated with ficolins.

4.3.5.1 Effect of GNA against pseudoparticles

To test the antiviral properties of lectins [330] against hepacivirus pseudoparticles, a purified lectin (GNA) known to bind to mannose sugar moieties on hepacivirus was used. GNA was preincubated with H77pp, J6pp, EqHVpp and VSVGpp for 1 h at room temperature. Dose dependent inhibitory effect of GNA on hepacivirus pseudoparticle infection was observed for HCVpp and EqHVpp but not J6pp (Figure 4-6). GNA was non-inhibitory to VSVpp (Figure 4-6).

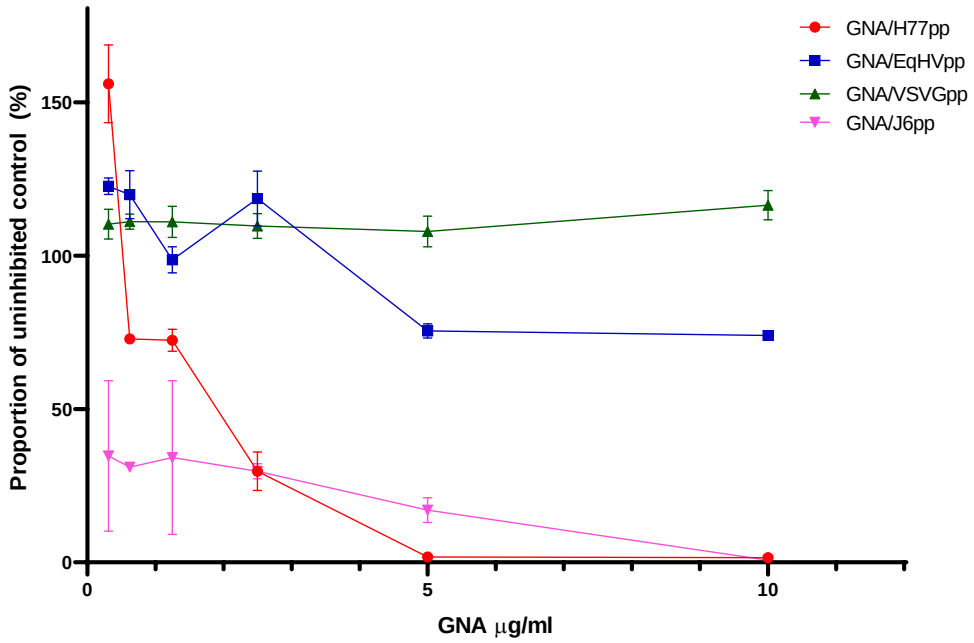

Figure 4-6: Antiviral effect of GNA against hepacivirus pseudoparticles

Different pseudoparticles; HCVpp (H77 and J6), EqHVpp and VSVGpp were used in this assay. GNA was incubated for 1 h at room temperature with serial dilutions of GNA starting with a neat concentration of 10 µg.mL^{-1} which were then added to Huh-7 cells. After 72 h, cells were lysed, and infectivity was determined by pseudoparticle-based luciferase activity in Huh-7 cells using a luminometer. Infectivity was normalised to uninhibited pseudoparticle (100%) and ΔE glycoprotein-deficient (0%) controls to determine the neutralisation activity of GNA. All conditions were performed in triplicates. This result is represented as the standard error of mean of one experiment.

4.3.5.2 Neutralisation of pseudotype virus infection by purified recombinant ficolins expressed by different mammalian species

The nickel affinity purified ficolin proteins from different mammalian species were used in the pseudotype virus neutralisation assay (Figure 4-7). A neutralisation assay was performed with a 5-fold dilution of preparations of FCNA, HumFCN2, GoFCN2 and a negative control (cells mock-transfected without an expression plasmid; NEG) starting with a dilution of 1/10. Neutralization of HCV-H77pp, EqHVpp, EBOVpp and VSVpp were performed. None of the ficolins gave a dose-dependent inhibition of viral entry. Although, the infectivity of H77pp, EqHVpp, and EBOVpp (Figure 4-7 A, B & C) was reduced by all the ficolins between 60-80%, this result can be said to be invalid as the cells-only control (NEG) had a comparable effect. The entry of VSVpp into Huh-7 cells was not inhibited, with 100% infectivity at all dilution factors of the protein (Figure 4-7 D).

In another attempt, the total protein quantified from the purified ficolin was normalised to a concentration of 200 $\mu g.mL^{-1}$. The result was the same with no neutralisation of the pseudoparticles and the negative control protein had the same effect (data not shown).

4.3.5.3 Effects of DMEM, Opti-MEM and PEI on the neutralisation assays of HCVpp

Due to the unexpected inhibition of virus infection by the cells-only preparation (NEG), the possible effect of fresh media (DMEM and Opti-MEM) used before transfection, the cells-only preparation (Opti-MEM following transfection) (NEG) and the transfection reagent (PEI) were investigated in this assay. Fresh DMEM and Opti-MEM were concentrated like the cells-only preparation (NEG) (section 3.2.8). A five-fold serial dilution of the fresh concentrated DMEM, Opti-MEM and the cells-only preparation (Opti-MEM following transfection) was performed from 1/5 to 1/3125, in triplicates, and preincubated with H77pp for 1 h at room temperature. DMEM inhibited

the infection of H77pp greater than Opti-MEM at a half maximal inhibitory dilution (ID_{50}) of 1/5 dilution.

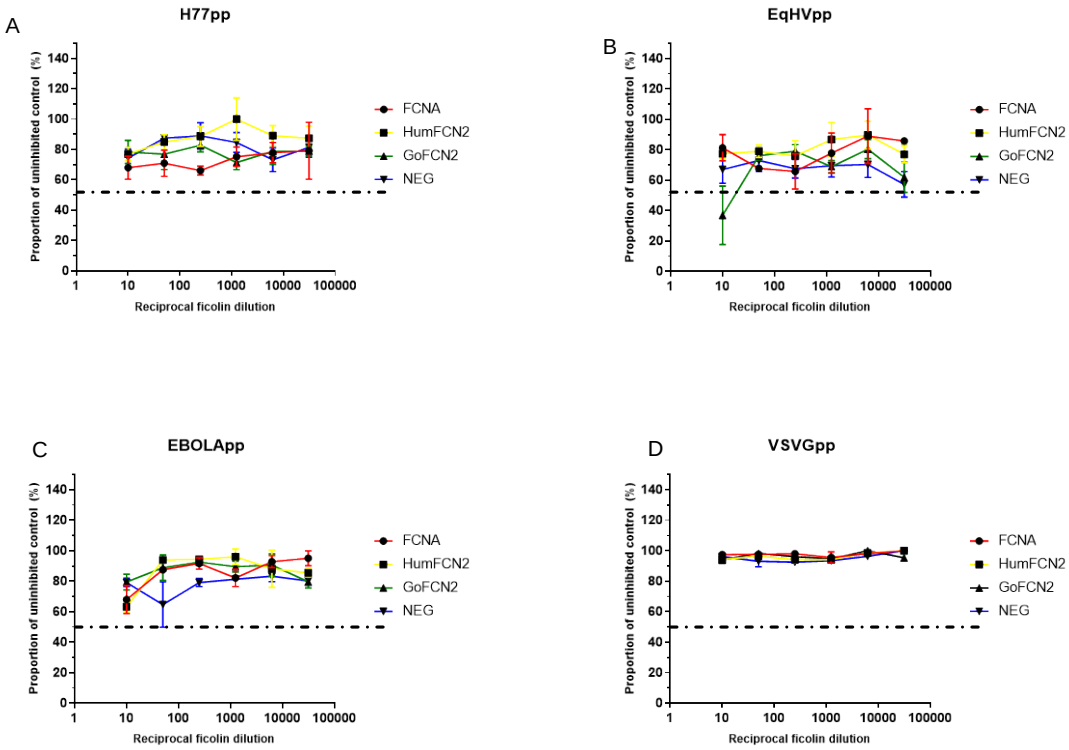

Figure 4-7 Neutralisation assay using retroviral pseudotypes representing different virus species and purified ficolin preparations

HCVpp and EqHVpp expressing E1/E2 derived from isolates A) H77 and B) PAA respectively, Ebola (C) and VSV-G (D) were incubated for 1 h at room temperature with serial dilutions of purified ficolins and added to Huh-7 cells. Infectivity was determined by pseudoparticle-based luciferase activity in Huh-7 cells using a luminometer. This was normalised to uninhibited pseudoparticle (100%) and ΔE glycoprotein-deficient (0%) controls to determine the neutralisation activity of ficolins from different mammalian species. All conditions were performed in triplicates. Error bars represent the standard error of the mean.

However, when fresh Opti-MEM was further concentrated to the lowest concentrate volume of 250 µL (Opti-MEM_250mrk) using the Amicon concentrator (section 3.2.8) before use in a neutralisation assay, it blocked infection as well as the cells-only preparation (Figure 4-8 A). Subsequently, the transfection reagent (PEI) was tested by producing cells-only preparation with or without PEI (OptiMEM- following transfection protocol with/without PEI). Two volumes of PEI (48 µL and 72 µL) were used in the neutralisation assay (Figure 4-8 B & C). Based on the total protein calculated from BCA assay, a 10-fold dilution factor starting from 1/10 was used in

the neutralisation assay. This was to avoid any possible effect of unidentified proteins, especially in the 'cell-only' preparation on entry of the pseudotypes. The cell only preparation which was transfected with PEI (48 µL) had the same pattern of inhibition as the cells-only preparation without PEI when tested against J6pp, see Figure 4-8 C, but was less potent against H77pp (Figure 4-8 B). Although H77pp and J6pp showed no difference in the overall susceptibility to cells-only preparations transfected with different volumes of PEI (48 µL and 72 µL) (Figure 4-8 B & C) used, there was a consistent inhibition at 60% at dilution factors of 1:100 and 1:1000 for both pseudoparticles.

Together, these results suggest that some components expressed by HEK 293T cells during culture greatly affect the entry of the pseudotyped viruses tested. Although the mechanism for this is not clear, the amount of inhibition of infectivity might bear some relation to the sequence of the glycoprotein genes tested.

4.3.5.4 Neutralisation of pseudotype virus infection by unpurified recombinant ficolins expressed by different mammalian species

Based on the result obtained from the neutralisation by purified ficolins above, the neutralising activity could not be attributed to the ficolin preparations as the medium arising from the cell-only preparation also resulted in the inhibition of pp entry, albeit the presence of ficolin in the preparations were low and could not be quantified as shown in the previous chapter. To investigate the ability of the purified ficolin proteins to inhibit infection of the hepacivirus pseudotypes into Huh-7 cells, the unpurified protein was also explored. The harvested ficolin supernatants were concentrated to 500 µL or 1 mL as described in section 3.2.8. For small-scale optimisation purposes, only the hepaciviruses were focussed on in this assay. A 5-fold dilution series was performed for the neutralisation assay, starting with a dilution of 1/5, performed using FCNA, HumFCN2, GoFCN2 and a supernatant from the cell-only control (NEG) against H77pp, EqHVpp and J6pp (Figure 4-9). Representative data from three

experiments showed that ficolins expressed by different mammalian species reduced infectivity of hepaciviruses by up to 50% (Figure 4-9 D).

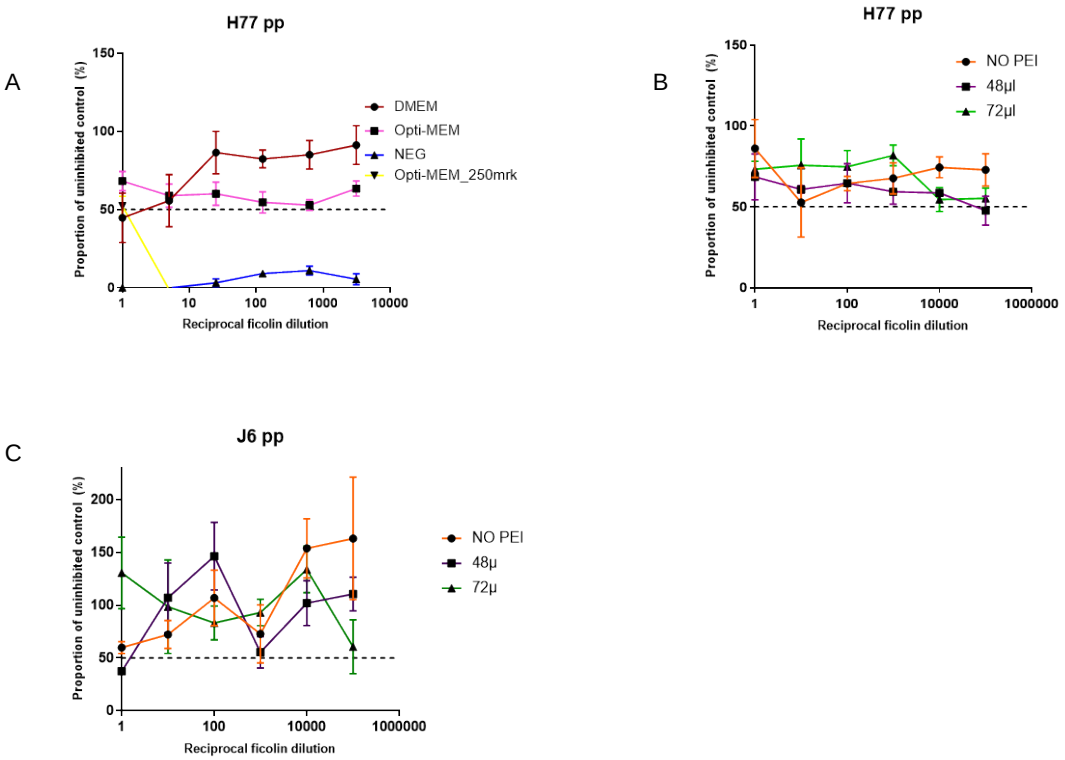

Figure 4-8 Neutralisation effects of concentrated DMEM, Opti-MEM and PEI on HCVpp entry into target cells (Huh-7 cells)

H77pp expressing E1/E2 glycoproteins was preincubated with concentrated DMEM, Opti-MEM, negative control and Opti-MEM concentrated to the lowest concentrate volume of 250 µL (250 mark) for 1 h at room temperature starting with the neat normalised protein at 0 and a serial dilution at 1/5. A) H77pp B) and J6pp (C) were preincubated with different 2 volumes of the transfection reagent (PEI) and no PEI for 1 h at room temperature starting from a serial dilution of 1/10. The sample transfected with 72 µL PEI is equivalent to the negative control (NEG) used for the other neutralisation assays in this study. Infectivity was determined by pseudoparticle-based luciferase activity in Huh-7 cells using a luminometer. This was normalised to uninhibited pseudoparticle (100%) and ΔE glycoprotein-deficient (0%) controls to determine the neutralisation activity of ficolins from different mammalian species. All conditions were performed in triplicates. Error bars represent the standard error of the mean.

Ficolin preparations containing HumFCN2 and GoFCN2 inhibited entry of the two HCV genotypes (H77 and J6) in a dose-dependent manner (Figure 4-9, A & C). EqHV was neutralised by FCNA and HumFCN2 but was not neutralised by GoFCN2 (Figure 4-9 B). Overall, all the ficolins expressed from different mammalian species showed

higher potency, with low and comparable ID_{50} values against J6 (Figure 4-9 D). Similar to previous assays, the cell-only preparation (NEG) had a similar effect. As much as this data looked promising, the cell-only preparation (negative control) (NEG) which was transfected with 72 uL of PEI and no expression plasmid was also inhibiting the entry of the pseudoparticles. This experiment was repeated several times and the negative protein showed dose-dependent inhibition still (data not shown).

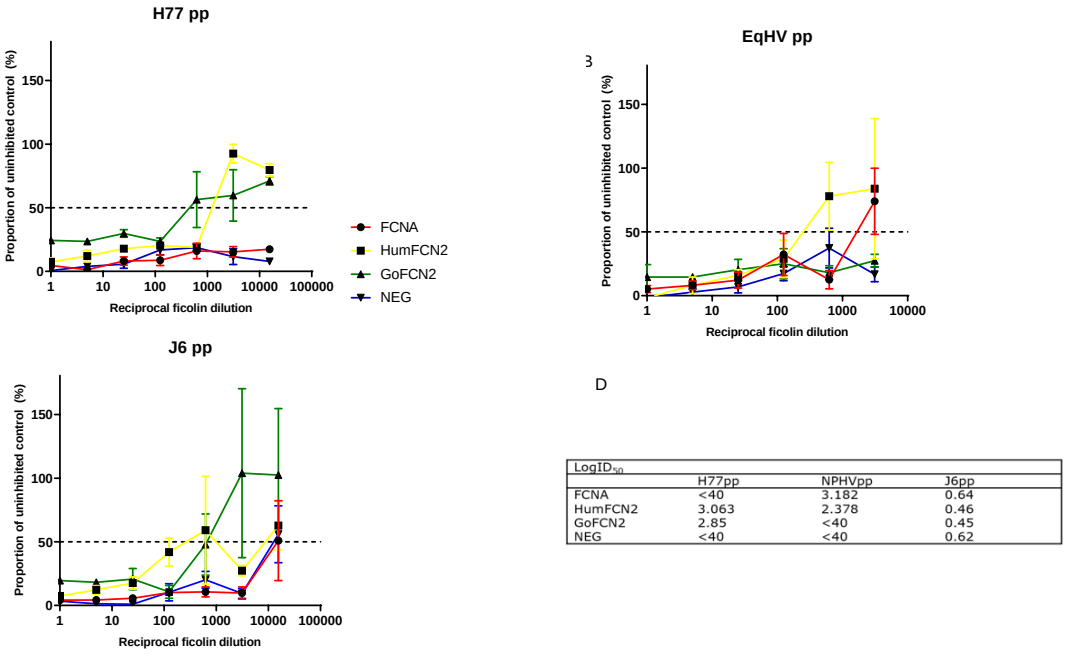

Figure 4-9: Neutralisation of hepaciviruses by cell culture preparations containing ficolins

HCVpp derived from clones H77 (A), EqHVpp (PAA) (B) and J6 (C) were preincubated with ficolins at the indicated dilution factors before infection of huh7 cells. Infectivity was determined by pseudoparticle-based luciferase activity in Huh-7 cells using a luminometer. This was normalised to uninhibited pseudoparticle (100%) and ΔE glycoprotein-deficient (0%) controls to determine the neutralisation activity of ficolins from different mammalian species. All conditions were performed in triplicates. D) shows ID_{50} calculated from the dose-dependent curves shown in A, B and C. Error bars represent the standard error of the mean.

4.3.5.5 Optimisation of neutralisation assays of pseudotype virus by recombinant ficolins expressed by different mammalian species

Although the proteins expressed after transfection have influenced the entry of viral pseudotypes, the assay was further optimised. In this assay, ficolin preparations were further diluted to start at a dilution series of 1/10 as against 1/5 used in the previous assay (section 4.3.5.4). To include the possibility of strain specificity, additional

experiments were performed using pp(s) possessing different glycoproteins. FCNB was also included to compare activity of other ficolin types (Figure 4-10). A five-fold serial dilution of unpurified recombinant ficolins expressed from different mammalian species (the starting material (S) in Figure 3-10 B) were preincubated with pseudoparticles of HCV (J6 and H77), EqHV, Ebola and VSV, each in triplicate. Measurement of the luciferase activity showed that pseudoparticles expressing E1/E2 were inhibited to different degrees by ficolins in a dose-dependent manner (Figure 4-10, A, B, C). At higher dilution, FCNA, FCNB and HumFCN2 blocked infection of Huh-7 by H77pp >90% while GoFCN2 inhibited in a dose-dependent manner, with a 50% inhibitory dose at a dilution of 1/1250 (Figure 4-10 A). Similarly, all recombinant ficolins had a dose-dependent neutralising effect, inhibiting J6pp infection by 50%; FCNA and HumFCN2 at 1/1250 and GoFCN2 at 1/250 dilution factor (Figure 4-10 C). In contrast, HumFCN2 and FCNB showed a strong and dose-dependent inhibition of EqHVpp and VSVpp (Figure 4-10 B & D). FCNA, GoFCN2 and the cell-only preparation/negative transfection control (NEG) all inhibited EqHV (range of 60 to 70%) after an unstable increase and decrease at different degrees. GoFCN2, FCNA and NEG did not affect infection of VSV pseudoparticles. FCNA, HumFCN2 and GoFCN2 enhanced the infection of pseudoparticles possessing the Ebola virus glycoproteins (Figure 4-10 E). Overall, this result provided some evidence that FCNB efficiently neutralised hepaciviruses better than HumFCN2 and the effect of the negative control was somewhat reduced with increased dilution. In spite of the distinct inhibition of the entry of hepaciviruses by FCNB and HumFCN2 in this result, the dose dependent effect of the negative control without expression plasmid persisted.

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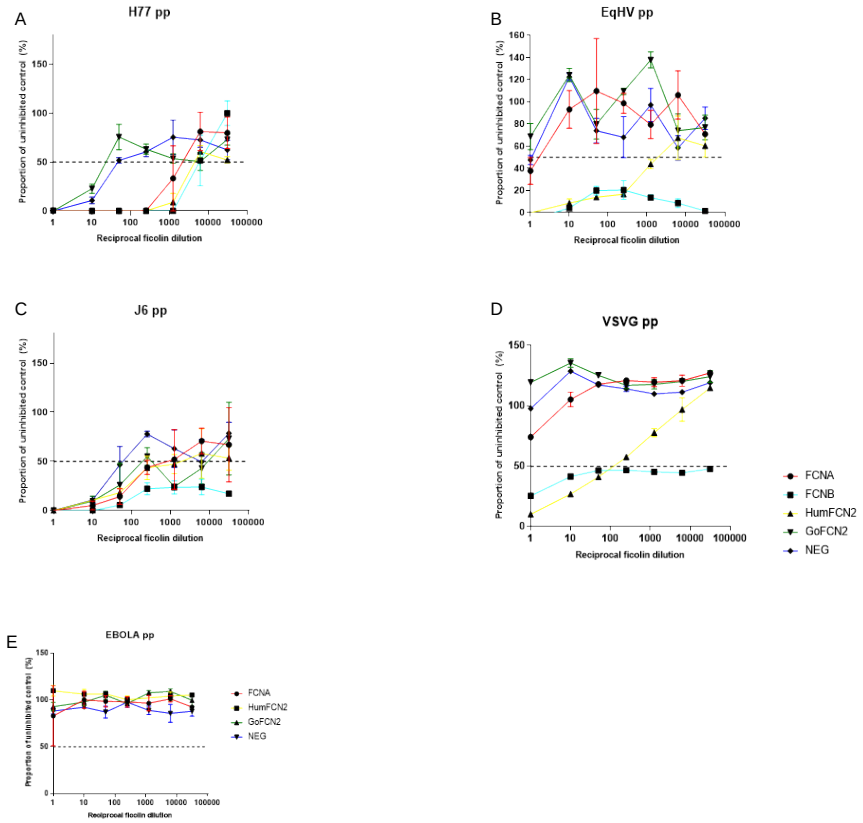

Figure 4-10 Optimisation of recombinant ficolins neutralisation of HIV-1 based pseudotype infectivity

HIV-based pseudotype particles expressing different E1/E2 glycoproteins (A, B and C), G protein (D) and Ebola surface glycoproteins (E) were preincubated with 5-fold serial dilution ficolins and negative control for 1 h at room temperature. Infectivity was determined by pseudoparticle-based luciferase activity in Huh-7 cells using a luminometer. This was normalised to uninhibited pseudoparticle (100%) and ΔE glycoprotein-deficient (0%) controls to determine the neutralisation activity of ficolins from different mammalian species. All conditions were performed in triplicates. Error bars represent the standard error of the mean.

4.4 **Discussion**

Hepaciviruses express two envelope glycoproteins E1 and E2, which mediate entry and are the target of pathogen recognition receptors produced by the host [153]. Both proteins are highly glycosylated, and while some glycans are conserved, others are variable between strains [145, 149, 331]. The glycans of E1/E2 bind to human ficolin-2, leading to the activation of the lectin complement pathway [31]. This is evidence that glycans serve as ligands for human ficolin-2 and possibly for ficolins expressed from other mammalian species as all ficolins show specificity for N-acetyl glucosamine

residues. Several members of the ficolin family have been identified (Chapter 1, also reviewed in [332]). Different studies have examined the role of human ficolin-2 as an important aspect in host innate defences against viral infections [29, 79, 86-88]. Other lectins including GNA (a plant lectin) have also been shown to have an antiviral effect against different virus cell cultures and pseudoviruses by interacting with glycans present on the surface of enveloped viruses leading to inhibition of viral entry and blockage of viral replication [330, 333, 334]. To date, there have been no published studies on the interaction of other types of ficolins other than human and different viruses. This study characterised the binding of ficolins to hepacivirus glycoproteins. The ability of ficolins from different mammalian species to inhibit virions from infecting target cells was investigated through neutralisation assays.

HCV has a narrow species tropism for humans, although chimpanzees are susceptible to experimental infection [335]. This has hampered the development of prophylactic vaccines and there is no evidence for an animal reservoir of viruses closely related to HCV [98]. Recently, EqHV in horses was identified as the closest relative to HCV [336] with approximately 50% sequence homology [95]. Interestingly, hepatotropism has been demonstrated for EqHV [98] meaning they could infect the hepatocytic cell line Huh-7, however, the function of their glycoproteins (E1/E2) by retroviral pseudotyped viruses have not been assessed. Given the close similarities between HCV and EqHV, a pseudotype entry assay was developed for EqHV. Subsequently, the ability of GNA or ficolins to inhibit virus infectivity of Huh-7 cells by pseudoparticles produced was determined. Using HCVpp as a model, a functional lentivirus particle in which the surface envelope glycoprotein gp160 is replaced by EqHV E1 and E2 glycoproteins (EqHVpp) was generated [153]. It was important to compare the quantity of plasmids versus the backbone used to avoid discrepancies (Figure 4-3), as the functionality of the EqHVpp produced was dependent on this. This is consistent with previous reports where reference infectious clones [247] have been used and particular variants did not tolerate the HCVpp entry assay [337]. To further

validate the susceptibility of Huh-7 cells to EqHVpp, harvested supernatants which have been processed through sucrose gradient were used to perform a western blot which showed bands with p24 HIV core, HCV and EqHV E1 and E2 envelope proteins (Figure 4-3 B). This result supports previous studies which demonstrated that these proteins are required for pseudotyped retroviral particles infection and that particles expressing E1/E2 protein do enter the target cells [251, 338]. Pelleting pseudoparticles through a sucrose cushion predicts glycoprotein incorporation by the detection of HIV-1 p24. Consistent with this study is a previous analysis of sucrose-gradient sedimented HCVpp preparations, where fractions containing infectious pseudoparticles were associated with detectable HIV-1 p24 [339].

HCV and EqHV are classified within the family *Flaviviridae* [340] whose members exhibit pH-dependence in cell entry [341, 342]. Consistently, results from this study (Figure 4-4 A) established that an inhibitor of endosomal acidification (bafilomycin) reduced the infectivity of HCVpp and EqHVpp. The HIV-based HCV pseudotyped viruses' pH dependence is in line with a receptor-mediated endocytic pathway of virus entry [251]. Viruses that enter the cells through this pathway can prevent premature fusion of the internal cellular membranes by synthesising their viral fusion glycoproteins in an inactive form [343]. Having validated the entry of HIV-EqHV particles, an MLV-EqHVpp was produced (Figure 4-5), as previous studies have shown that the species of packaging construct can influence infectivity between different HCV glycoproteins [247]. Interestingly, most HCV studies have utilised the HIV-1 [251] or MLV backbones [225]. The retroviral pseudotype packaging constructs to use in each experiment are determined by the infectious titre and the ability to give reproducible infectivity [344]. Here, the MLV-EqHVpp and HCVpp were found to have little or no infection (Figure 4-5) compared to when the hepaciviruses were pseudotyped with the HIV core, suggesting that incorporation of hepacivirus glycoproteins into retroviral particles depends on the assembly of the packaging virus.

Having shown that the process of HCVpp and EqHVpp entry into target cells is receptor-mediated [154, 345, 346] and can be activated in a pH-dependent fashion, the inhibition of this process was explored. Here, the ability of lectins including GNA and ficolins to inhibit the infection of target cells by hepaciviruses was determined. GNA can inhibit HCVpp and HCV whole genome entry [347] and other enveloped viruses such as HIV, influenza virus and Simian immunodeficiency virus (SIV) [348-350]. This study verified that GNA inhibited hepacivirus entry in a dose-dependent manner (Figure 4-6) by binding to the N-linked glycans on the surface of the virus. This further validates the functionality of the pseudotyped particles produced and ficolin antiviral functionality was subsequently determined. The unpurified protein preparation inhibited the viral pseudoparticles better than the purified protein preparation, albeit the observed inhibitory effect of the untransfected cell-only control (NEG). Two of the most commonly used HCV strains (H77 and J6) were used in this study. Ebola Zaire (Ebolapp) whose infection is reduced or enhanced by human ficolins and MBL [329] was included as a control to examine the activity of mouse or gorilla ficolin-2 compared to the human ficolin-2. VSV-G pseudotyped lentivirus was used as a positive control for viral entry and infection because of its broad tropism [87, 351]. The purified protein preparation did not affect the infection of HIV-VSV-G (Figure 4-7 D). However, murine FCNA inhibited entry of H77pp and EqHVpp better than human ficolin-2 and gorilla ficolin-2 with the untransfected cell only negative control having the same effect, making this result inconclusive (Figure 4-7). The unpurified ficolins inhibited the infection of Huh-7 cells by H77pp, EqHVpp, and J6pp (Figure 4-9). The inhibition by hepaciviruses in this study is consistent with previous studies where MBL from different HCV genotypes inhibited infection to differing extents [149]. In contrast, the effect of an untransfected control wasn't shown, and this continues to show similar effects with ficolins in this study, hence the specificity of ficolin mediated neutralisation could not be determined. This effect was consistent after conducting three independent experiments and was somewhat reduced when

the protein preparations were diluted from a starting dilution of 1/5 (Figure 4-9) to a starting dilution of 1/10 (Figure 4-10) in the neutralisation assay.

The unexpected neutralising effect of the negative control (NEG) was investigated by using alternative approaches to generate the recombinant protein in HEK 293T cells. Fresh concentrated DMEM, Opti-MEM, different concentrations of PEI and no PEI in transfection experiments were implemented when expressing the recombinant ficolins, to see if this was an effect of just growing the HEK cells in culture. Fresh concentrated Opti-MEM that was used for HEK cell growth for a 72 h period inhibited H77pp infection up to 50%, while the negative control and highly concentrated Opti-MEM totally blocked infection (Figure 4-8). A possible effect of a non-specific protein or chemical in the Opti-MEM/NEG might influence the inhibition of H77pp, although this remains to be confirmed. However, the effects of PEI (transfection reagent) and no PEI show a different effect in H77pp and J6pp. While the former's infection was inhibited by the sample that had not been treated with PEI during the transfection phase (Figure 4-8 B), the infection of the latter was not affected (Figure 4-8 C). All this put together, a non-specific protein detected by western blot using an anti-His-tag antibody (Abcam) (Figure 3-3) was speculated to influence this unexpected effect of the negative control protein preparation. This might seem similar to an endogenous non-specific 60 kDa protein which was defined as a transcription regulator YY1 detected by an anti-His antibody [352] after subjection to a mass spectrometry analysis [353]. It was confirmed that the protein had 11 histidine residues without known functions [354, 355]. A subset of YY1 carries GlcNAc moieties, making it also exist as a glycoprotein [356]. Although the cell-only negative control in this study might be like YY1, this can only be confirmed by performing a western blot analysis with an anti-YY1. A limitation of this study was that ficolins from different species were only expressed in 293 T cells and not from other cell lines such as CHO, liver sample or myeloid cells, which might differ in host cell factors and their ability to bind the viral particles. Also, the cytotoxicity of the cell supernatants for target cells (Huh-7) was not

at any point measured. However, given that VSV-G-mediated pseudoparticle entry was not inhibited by these samples, it is plausible that cells were healthy and replicating, and able to express the reporter gene. Further studies are needed to determine whether a similar mechanism accounts for the inhibition of HCV and EqHV infection by ficolins from different species investigated in this study and the negative control in CHO cells or a different cell-line.

Reducing the amount of protein diluted from five to ten-fold dilution gave the best result for the unpurified protein. This was optimised for all the pseudoparticles investigated in this study and the negative control effect was reduced. Consistently, the recombinant mouse ficolin B inhibited infection of HCVpp, EqHVpp, J6pp and VSV-Gpp better than all other recombinant ficolins. Interestingly, FCNA and human ficolin-2 had the same pattern of neutralising potency. This is plausible as FCNA is the orthologue of human ficolin-2 (As reviewed in chapter 1, section 1.3.2) although phylogenetically, each protein is unique (chapter 1, section 1.8 and [17]). Pseudotype infectivity assays previously showed that H77pp and J6pp formed functional glycoproteins [328]. However, this is the first time the EqHV pseudotype infectivity has been assayed. All neutralisation potency shown by human ficolin-2 in this study is consistent with other studies where recombinant ficolin-2 neutralises HCV and other viruses [29, 90]. Furthermore, all ficolins expressed did not display the ability to modulate Ebolapp infection. This is similar to the findings from a previous study that showed that human ficolin 1 enhanced Ebola infection amongst all other ficolins [329]. The authors confirmed the unique binding specificity of human ficolin 1 to sialylated ligands [357]. Human ficolin-2 and FCNB also inhibited entry of VSVpp, with other ficolins having no effect. Comparing the binding and inhibiting activities of other mammalian ficolins to that of human ficolin-2, it becomes clear that while one ficolin shows high viral inhibition, another ficolin achieved inhibition of the pseudotyped virus only to a low extent.

Although the untransfected cells-only negative sample continues to inhibit hepaciviruses in this study, results from this study predict that infection of HCV (H77, J6 strains), EqHV and VSV pseudoparticles were inhibited by ficolin proteins cloned from different mammalian species. It is not clear what property is common to all these viruses that determine their sensitivity to ficolin neutralisation. Evidence from previous studies indicated that differences in the number of potential glycosylation sites occupied by glycans have been linked to the entry of HCVpp [145] and HCVcc [146]. Binding of ficolins to HCVpp and subsequent inhibition of infectivity illustrates that ficolins can block infectivity of the hepaciviruses tested here. Previous studies by our research group and others have previously shown that neutralisation of HCV and influenza A by MBL was influenced by the extent of the glycosylation of the viral glycoproteins and the spatial arrangement of the N-linked glycans [149, 358]. In this study, a defined relationship between numbers of N-linked glycan sequons and ficolin neutralization does not exist for hepacivirus pseudoparticles tested. However, human ficolin-2 has been illustrated to effectively neutralise the entry of HCVpp and HCVcc representing genotypes 1, 2, 3 and 4. This was consistent with a role for conserved E1/E2 glycans in HCV entry [90]. Additionally, a preliminary study was carried out to characterise the glycosylation sites of the HCV E1/E2 glycoproteins required for human ficolin-2 interaction (unpublished data). This was achieved by making glycan knockout (GKO) library using multiple random mutations at HCV E2 glycosylation N-linked sites, especially those that have been shown to be essential for HCV entry [117, 145, 359]. While no specific glycosylation sites of interaction with human ficolin-2 was identified, data supported previous findings [145] of HCV E1/E2 glycosylation sites in HCV entry. Hence, the pseudoparticles produced in our research group might have the right spatial arrangement required for ficolin binding since they seem to be neutralised by human ficolin-2 in the same manner as the HCVcc. It is plausible that the specificity of ficolin antiviral activity might be associated with glycans on the surface of their glycoproteins. This antiviral activity is achieved by blocking the entry of virions into host cells. Thus, the neutralising activity of ficolins can be said to be

biotherapeutic, making them better therapeutic agents due to specificity for hepacivirus glycoprotein E1/E2. Human ficolin-2 has been shown as a biotherapeutic agent. Here, it is shown that mouse ficolin B has a better neutralising potency than human ficolin-2, except that the negative control also had a neutralising effect. This study proposes the possibility of a chimera of these two ficolins (human FCN2 and FCNB) as antiviral therapy for hepaciviruses as compared to a previous study where a combination of human MBL and ficolin -2 was made for influenza virus as well [86].

Ficolins play a crucial role in innate immunity by activating the complement system after binding to carbohydrates on the surface of a microbial pathogen [360]. Human ficolin-2 binding to N-glycans of E1 and E2 glycoprotein of HCV activates the lectin complement pathway and mediate cytolytic activities [31]. It was further confirmed that the human ficolin-2-MASPs complex were closely related to disease progression like MBL/MASP-1 activities [361, 362] suggesting that measurement of human ficolin-2 concentrations in HCV patients might serve as a diagnostic molecular marker for disease progression in HCV infection. However, the human ficolin-2 MASPs complex bind lipoteichoic acid from clinically important bacteria such as Streptococcus aureus and activate C4 [30]. Indeed, the activities of C4 in HCV correlate with a successful response to therapy [191]. Additionally, recombinant mouse ficolins activate complements [317]. Although a complement activation assay has not been explored in this study, it is plausible to speculate that the functional ficolins from different species expressed in this study can activate the complement system; however, more investigations are required to confirm this speculation.

4.5 **Conclusion**

Given the genetic closeness between HCV and EqHV, this study showed that EqHV shares entry characteristics with HCV. To the best of my knowledge, this is the first time an entry model was designed for EqHV and this will facilitate studies about hepacivirus evolution, immunity and pathogenesis. The development of hepacivirus

pseudotype particles to assess antiviral potency of molecules, such as lectins targeting viral entry, has made it possible to develop different inhibitors. Inhibitors of entry such as GNA and ficolins are potential antiviral therapy for HCV. Despite the development of new DAAs, there are concerns about drug resistance, high cost, and worldwide accessibility; hence effective vaccines and antiviral inhibitors remain an important need. There is also a mechanistic difference between ficolins as potential inhibitors and DAAs. The latter inhibit viral replication, while ficolin has the potential to prevent HCV and hepacivirus entry into targeted cells.

This study attempted to interrogate if ficolins can inhibit virus entry by interacting with hepacivirus envelope glycoproteins (E1/E2). However, the challenge of the non-specific inhibition observed by the negative control (cell-only preparation) showing the same inhibitory effect remains unresolved. Further studies are required to show the specificity of the inhibitory effect by each ficolin when compared to the negative control. Despite these issues, the data presented here showed that recombinant FCNB inhibits HCV, EqHV and VSV pseudoparticles more potently than human ficolin-2, and this suggests that mouse ficolin is a potent entry inhibitor. Interestingly, none of the ficolins affected the entry of Ebola virus. It can be speculated that the ficolins from different mammalian species expressed in this study may lead to complement activation after binding to the envelope glycoprotein of the viruses. In the absence of the inhibitory effect of the negative control, a recombinant chimeric lectin comprising human and mouse ficolins could be proposed as a new antiviral inhibitor as this may surpass the activity of human ficolin-2 leading to significantly higher binding affinity to viral glycoproteins. This could be a suitable candidate for developing new anti-antiviral therapy.

5 General Discussion, Future Recommendations and Final Conclusion

5.1 General discussion and Future Recommendations

Human ficolin-2 has been demonstrated to bind to HCV virions and inhibit infection. However, HCV persists in the presence of a constitutive expression of this protein in the serum of infected patients [363], suggesting that the virus may adapt to avoid recognition by this host immune effector. Given the highly polymorphic nature of ficolin proteins, it was investigated whether ficolins isolated from other mammalian species possessed a more potent anti-HCV effect than the human form of FCN2.

This work aimed to characterise ficolins expressed in a range of mammalian species and define their role as direct immune inhibitors, as compared to human ficolin-2 in hepatitis C virus and non-primate/equine hepacivirus infections. Achieving this did not just require the characterisation of ficolins, but also HCV and EqHV. NHP ficolin-2 gene constructs were created from the liver for the first time. Moreover, the development of EqHVpp enabled the functional investigation of EqHV entry, which has been shown to be the closest relative to HCV and might serve as a surrogate model for HCV.

5.1.1 Evolution of Ficolin Genes

In humans and non-human primates, three ficolin genes (*FCN1, FCN2* and *FCN3)* have been identified, while the equivalent *fcna* and *fcnb* are found in mice. The *FCN3* gene in both rat and mouse ficolin is a pseudogene. The aim here was to understand the phylogenetic and inter-molecular relationship of mammalian ficolins and how this affects the structure and function of their protein. Ficolins expressed by different mammalian species were successfully cloned, sequenced, and their coding region was aligned. The human ficolin and equivalent non-human primate proteins showed high homology while the mouse ficolins varied. The homology between human and

non-human primate ficolin shows that they have an ancient ancestor. Further analysis revealed that most of the variations were found in the FBG domain; the part of the protein responsible for binding to ligands. The variations may affect the binding activity of each protein; hence it is plausible to conclude that the binding of each recombinant ficolin may be different because of the uniqueness of each protein. However, more investigation of the non-synonymous variations found in the FBG is required. Due to the polymorphic nature of ficolins, *in vitro* assays and computation variant analysis will guide the selection of genetic variants that might be likely functional.

5.1.2 Analysis of Recombinant Ficolins

All ficolins from the mammalian species investigated were successfully expressed in HEK 293T cells. The expressed recombinant gorilla ficolin-2 and mouse ficolins displayed the same characteristic oligomeric structure as recombinant human ficolin-2. Although the recombinant ficolins were His-tagged, it was difficult to purify them to a high degree, due to low yield during expression. This is in contrast with a previous study where human ficolin-2 was FLAG-tagged [193]. To acquire a higher expression level of the recombinant proteins in future studies, the expression could be optimised- with the use of bioreactors, growing HEK cells in suspension to increase yield and improve transfection efficiency by using different plasmid concentrations or the 293T cells could be allowed to grow for a longer period before harvesting the supernatant since a reduced serum media (Opti-MEM) was used. Alternatively, other cell lines, such as CHO cells, could be used instead of HEK 293T. HEK cells were used because they have been used in our research group for the expression of recombinant proteins, including human ficolin-2 [90, 149, 294]. Additionally, HEK 293T shows the highest level of PEI- mediated transfection amongst other mammalian cell lines [364, 365]. CHO cells have been used in the expression of mouse and non-human primate ficolins [11, 34, 304, 309]. Better still, to overcome the lack of control over the quality and quantity of plasmid DNA delivered to the cell, an *in-vitro* system could be

generated to deliver the recombinant gene of ficolins into a stable mammalian cell line for expression [302]. Lentiviral vectors have been used to develop stable cell lines for protein expression [366-368]. Although the purification was successfully optimised, the purified protein yield was still low. Further purification steps such as size exclusion chromatography and affinity chromatography using GlcNAc or CysNAc can be explored. The greatest limitation of this study was not being able to quantify the protein from all the ficolins from different mammalian species or to purify the protein prep to sufficient purity to prevent problems with the background effects of culture components.

Successful functional assays for the recombinant ficolins expressed by different mammalian species showed their binding ability to acetylated BSA in a calcium-dependent manner. However, there have been controversial studies whether the binding of human ficolin-2 to GlcNAc is calcium-dependent or independent [32, 48, 319]. For future studies, investigating the ability of the expressed recombinant ficolin to activate the lectin pathway of the complement system will show a major function of ficolins. Regarding these findings, it will be interesting to investigate the single nucleotide polymorphisms of ficolins from other mammalian species as this has been shown to affect the serum level and binding activity for human ficolin-2 [72, 74]. In this study, the recombinant NHP ficolin Strep-tag construct which has been successfully cloned into an expression vector should be further investigated in the future. This can be expressed in a human cell-line, detected in western blot using a Strep-tag antibody and can be purified using a Strep-tag system by affinity chromatography.

5.1.3 Ficolin Binding to hepacivirus E1/E2 Glycoproteins

Human ficolin-2 has been demonstrated to bind to HCV glycoproteins. The interaction of human ficolin-2 with viral glycoproteins is based on the ability of human ficolin-2 to bind to acetylated ligands, more precisely, the N-acetylated residues. This interaction takes place through the fibrinogen domain of human ficolin-2. Therefore, it was

hypothesised that ficolins from other mammalian species should have the same binding capacities to the E1/E2 glycoprotein. The interaction between ficolins expressed from different mammalian species and HCV E1/E2 heterodimers were broadly comparable. This was a key functional evidence for both ficolins and viral glycoproteins suggesting a therapeutic effect of ficolins against HCV infection, as no further analysis can be done without this confirmation. Binding of human ficolins can be attributed to the high mannose oligosaccharide on the surface of E1/E2 possessing $GlcNAc_2$ stem [117, 324] leading to activation of complement [369, 370]. For future studies, it will be interesting to show the binding of ficolins to authentic HCV particles in patients' sera and EqHV particles in horses' sera. This could be achieved by immunoprecipitation techniques which will help pull down HCV/ficolin or EqHV/ficolin complexes. Successful immunoprecipitation of EqHV and HCV from serum have been reported [237, 371, 372].

5.1.4 EqHV as Potential Model for HCV

HCV shows a narrow tissue tropism, which has hindered the development of small animal models for vaccine studies *in vivo* over the years. Chimpanzees have been the only immunocompetent animal model [373]. However, there has been a challenge with this model because of costs of maintenance for scientific research, availability and public resistance [374]. This limitation has led to further establishment of HCV infected mouse models either as humanised mice with only human hepatocytes or humanised mice with a human immune system and hepatocytes [375-377] although this model has their limitations as well. Hence a new model is of uttermost importance. EqHV has recently been found to be the closest related virus species to HCV, and this will further increase the established knowledge about hepacivirus-host interactions. Several studies have demonstrated similarities between EqHV and HCV as reviewed in Chapter 1 section 1.11.8. This study shows the development of an *in vitro* system for the study of EqHV entry. EqHVpp retroviral particles displaying E1/E2

heterodimers were successfully produced. Like HCVpp, EqHVpp entry is strictly E1/E2 dependent and was easily detected in these assays by measuring luciferase activity. This is interesting as EqHV does not appear to display the restricted host tropism exhibited by HCV as they have been shown to infect equines and canines. The fusion of viral and cell membranes is induced by a low pH. This was confirmed as glycoproteins from EqHV mediated entry into a human hepatocyte cell line (Huh-7) and this entry was pH-dependent. Similarly, HCV entry has been confirmed to be induced by low pH. Now that an EqHVpp system has been developed, more studies are required to identify if the HCV coreceptors are also required for EqHV entry as this will aid the understanding of binding, attachment and internalisation of the virus. Interestingly, ficolins were able to bind to glycans associated with the EqHV E1/E2 glycoproteins, and their role as an antiviral agent during EqHV infection can now be considered as comparable to HCV.

5.1.5 Ficolin Functions in Hepaciviruses and other Viral Infections

Ficolins are pattern recognition receptors that bind to carbohydrate-based pathogen-associated molecular patterns on the surface of viruses and other microorganisms leading to the activation of complement. Human ficolin-2 has been shown to interact with viruses including HCV through the N-linked glycans on viral envelope glycoproteins [29, 90, 378]. Therefore, human ficolin-2 can limit HCV infection as part of the first line of immune defence before the development of specific adaptive immunity. However, no direct antiviral effect on hepaciviruses has been described for ficolins expressed from other species. Consequently, the potential of ficolins from mammalian species to bind hepaciviruses was evaluated by the inhibition of infection in the HCV and EqHV pseudoparticles system. Ficolins from the mouse ficolin seem to have inhibited HCV and EqHV pseudoparticle infectivity to different degrees. However, the negative control had the same effect. More precisely, FCNA, which is an orthologue of human ficolin-2, inhibited HCVpp (both strains H77 and J6) in a dose-

dependent manner at the same degree, and completely inhibited infection of H77 as human ficolin-2. This is quite interesting as it has been proposed that the neutralisation efficacy of lectins is determined by differences in E1/E2 glycosylation between HCV genotypes, with genotypes that are more heavily glycosylated being more susceptible to neutralisation [149]. Surprisingly, gorilla ficolin-2, which has 90% homology to human ficolin-2, did not neutralise as much as the other ficolins. Interestingly, all the recombinant ficolins expressed in this study did not inhibit the infection of Ebola pseudoparticles, which is consistent with other studies where lectins have been shown to enhance the infection of the virus [87, 329]. However, human ficolin-2 and FCNB did inhibit VSV-Gpp with the former having a greater neutralising potency in contrast to a previous study where VSV-Gpp was not inhibited by human ficolin-2 [193]. Overall, FCNB had a better inhibitory effect compared to human ficolin-2. This study provides suggestive data that mouse ficolins may neutralise hepaciviruses better than human ficolin-2, but until the problem with the inhibitory effect of the negative control is resolved, this cannot be stated with any certainty. Further studies on the effect of the untransfected control (NEG) by performing affinity binding studies with GlcNAc to compete for interactions between the ficolins and the E1/E2 glycoprotein could provide insights on the specificity of the inhibitory effect seen. There is a potential of a high amount of N-glycan on the surface of HEK 293T (transfection cell line) which plays an important role in cell survival and proliferation [379]. Hence, the inhibitory effect seen by the untransfected control might be cellular proteins binding to the target cell line and this can be avoided by treating the NEG sample with tunicamycin or N-glycosidase F (PNGase F) to remove N-glycosylation as shown by other studies [380, 381].

5.1.6 Ficolins as antiviral therapy

Some antiviral drugs designed against HCV infection have been aimed at the entry of the virus into the cells, blocking the replication and disrupting the viral assembly inside

the cells. Thus, these drugs can prevent the formation and reproduction of the virus rather than killing the virus [382, 383]. Here, hepacivirus entry may be a target for therapeutic intervention in chronic infection. Different antiviral drugs have been shown to inhibit HCV entry [384], and may prove useful as clinically relevant entry inhibitors. They have prophylactic properties and can be combined with other agents to show synergistic effect [384]. Human ficolin-2 is classified as an entry inhibitor because of its ability to bind to E1 and E2 during HCV infection [90]. Therefore, the ability of ficolins from different mammalian species to neutralise hepacivirus infectivity may be an important therapeutic vaccine development agent. This study suggests that mouse ficolin neutralised HCV better than human ficolin-2, but until the inhibitory effect of the negative control is resolved, this finding remains inconclusive. This suggests that HCV has evolved in the presence of human ficolin-2 to become somewhat resistant to the action of this protein. Consequently, in the absence of a transfected negative control effect, a novel chimeric recombinant protein containing domains of human ficolin-2 and mouse ficolin B could be produced, which might have potent antiviral properties. However, further investigation will be required to understand anti-hepacivirus infection mechanism of mouse ficolin B in mice and whether overexpression of this ficolin *in vivo* might elicit any form of autoimmunity.

The possible role of administering ficolins from different mammalian species as an antiviral/foreign protein into the human system must be cautiously considered. For ficolins to be used as antivirals clinically, this will depend on some physiological factors which are; bioavailability, toxicity and immunogenicity, routes of administration and affordability [333]. Therefore, improving the structure of a protein can enable its clinical utility. This is evident in different studies which have either designed more potent analogues, modified the protein to reduce immunogenicity or even swap domains, all to improve their suitability for clinical development [86, 385-387].

5.2 **Final Conclusion**

This study attempted for the first time to isolate ficolins from different mammalian species as recognition molecules for viruses. Hence, extensive effort was put into optimising the production of functional recombinant His-tagged ficolin proteins to facilitate these investigations. While expression was successful, purification of the protein produced very low yields and will require further optimisation. One of the greatest limitations of this study was not being able to quantify the amount of protein in each ficolin with a more specific antibody for each species, or to purify these proteins to sufficiently high degree of purity that allows in-depth analysis of their biological activity. Ficolins are types of lectins that offer many challenges and opportunities based on what is currently known about their unique functions. Understanding this protein is crucial in its effects on viral infections [18].

There is some evidence that the original hypothesis that species-specific differences in virus recognition by PRRs might be correct. HCV has evolved solely in human hosts for a prolonged time, always in the presence of human ficolin-2 in the serum. Hence, the virus may have adapted to persist in the presence of this protein. Consistent with this, our study suggests that mouse ficolin B recognised/neutralised HCV much better, although the negative control inhibitory effect remains unresolved. Generation of novel recombinant chimeric proteins derived from non-human ficolin species and humans may provide insight into the divergent evolution of these genes in mammals and their function in recognition of viruses. These proteins might also serve as a potential therapeutic anti-viral agent.

This is the first time an *in vitro* cell culture system has been developed to study EqHV as it is the closest relative to HCV. This study has attempted to produce infectious EqHVpp, which has shown some level of functionality in the hepacivirus entry pathway. These findings showed that HCV and EqHV might share common strategies for the early stages of their life cycle in terms of attachment and entry. Given the close

relatedness between EqHV and HCV, this study presents more understanding of the hepacivirus characterization and can be used to determine associated immunity by performing complement activation assays through the lectin complement pathway.

In conclusion, this study has characterised ficolins of different mammalian species that were expressed in cell culture and their roles in hepacivirus infections. Additionally, a foundation has been laid for EqHVpp production which can be used for the study of fusion mechanisms of the virus. This will give insights on using an animal model to study hepacivirus infections in their natural host.

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7 Appendix

7.1 A schematic representation of colobus and Gorilla ficolin with primers shown

A. 5’
CACCATGGAGCTGGACAGAGCTGTGAGGGTCCTGGGCCCTGCCAC
CCTGCTGCTCACTTTCCTGGGCTTGGCCTGGGCTCATCATCACCATC
ACCATGTCCAGGCGGCAGACACCTGTCCAGAGGTGAAGGTGGTGG
GCCTGGAGGGCTCTGACAAGCTCACCATTCTCCGAGGCTGCCCGGG
GCTGCCCGGGGCCCCTGGGCCCAAGGGAGAGGCAGGCACCAATGG
AAAGAGAGGAGAACGCGGCCCCCCTGGACCTCCTGGGAAGGCAGG
ACCACCTGGGTCCAAGGGAGCACCTGGGGAGCCCCAGCCATGCCT
GACAGGCCCACGCACCTGCAAGGACCTGCTAGACCGAGGGCACCT
CCTGAGCGGCTGGCACACCATCTACCTGCCTGACTGCCGGCCCCTG
ACTGTGCTCTGTGACATGGACACGGACGGAGGGGGCTGGACCGTTT
TCCAGCGGAGGGTGGGCGGCTCCGTGGACTTCTACCGGGACTGGG
TTGCCTACAAGCAGGGCTTCGGCAGTCGGCTGGGGGAGTTCTGGCT
GGGGAACGACAACATCCACGCCCTGACCGCCCGGGGAACCAGCGA
GCTCCGTGTAGACCTGGTGGACTTTAAGGACAACCACCAGTTTGCTA
AGTACAGATCGTTCAAGGTGGCCGACGAGGAGGAGAAGTACAATCT
GGTCCTGGGGGCCTTTGTGGAGGGCAGTGCGGGTGATTCCCTGAC
GTCCCACAACAACAACTCCTTCTCCACCAAAGACCAGGACAATGACC
TTAACACCGGAAATTGTGCTGTGACGTATCAGGGAGCTTGGTGGTA
CAGAACCTGCCATGTGTCAAACCTGAATGGTCGCTACCTCAGGGGG
GCTCATGACAGCTTTGCAAATGGCATCAACTGGAAGTCGGGGAAAG
GATACAATTACAGCTACAAGGTGTCAGAGATGAAGGTGCGACCTGC
CGGGGGGCAGCTGGAGCCACCCGCAGTTCGAAAAATAG 3’

Primers;

P1: 5’ CACCATGGAGCTGGACAGAGCTG 3’
P2: 5’ ATGGTGATGGTGATGATGAGCCCAGGCCAAGCCCAGG 3’
P3: 5’ CATCATCACCATCACCATGTCCAGGCGGCAGACACC 3’
P4:

5’
CTATTTTTCGAACTGCGGGTGGCTCCAGCTGCCCCCGGCAGGTCGCACCT
TCAT 3’ or CTAGGCAGGTCGCACCTTCAT (Without streptag)

B. 5’
CACCATGGAGCTGGACAGAGCTGTGGGGGTCCTGGGCTCTGCCAC
CCTGCTGCTCACTTTCCTGGGCATGGCCTGGGCTCATCATCACCATC

ACCATCTCCAGGCGGCAGACACCTGTCCAGAGGTGAAGATGGTGG
GCCTGGAGGGCTCTGACAAGCTCACCGTTCTCCGAGGCTGTCCGGG
GCTGCCTGGGGCCCCTGGGCCCAAGGGAGAGGCAGGCACCAATGG
AAAGAGAGGAGAACGCGGCCCCCCTGGACCTCCTGGGAAGGCAGG
ACCACCTGGGTCCAACGGAGCACCTGGGGAGCCCCAGCCGTGCCT
GACAGGCCCGCGCACCTGCAAGGACCTGCCAGACCGAGGGCACTT
CCTGAGTGGCTGGCACACCATCTACCTGCCCGACTGCCGGCCCCTG
ACTGTGCTCTGTGACATGGACACGGAAGGAGGGGGCTGGACCGTTT
TCCAGCGGAGGGTGGATGGCTCCGTGGACTTCTTCCGGGACTGGG
CCACGTACAAGCAGGGCTTCGGCAGTCGGCTGGGGGAGTTCTGGC
TGGGGAACGACAACATCCACGCCCTGACTGCCCAGGGAACCAGCGA
GCTCCGTGTAGACCTGGTGGACTTTGAGGACAACTACCAGTTTGCTA
AGTACAGATCATTCAAGGTGGCCGACGAGGCGGAGAAGTACAATCT
GGTCCTGGGGGCCTTCGTGGAGGGCAGTGCGGGTGATTCCCTGAC
GTTCCACAACAACCAGTCCTTCTCCACCAAAGACCAGGACAATGATC
TTAACACCGGAAACTGTGCTGTGATGTTTCAGGGAGCTTGGTGGTA
CAAAAACTGCCACATGTCAAACCTGAATGGTCGCTACCTCAGGGGG
ACTCATGGCAGCTTTGCAAATGGCATCAACTGGAAGTCGGGGAAAG
GATACAATTATAGCTACAAGGTGTCAGAGATGAAGGTGCGACCTGC
CGGGGGCAGCTGGAGCCACCCGCAGTTCGAAAAATAG 3’

Primers;

P1: 5’ CACCATGGAGCTGGACAGAGCTG 3’

P2: 5’ ATGGTGATGGTGATGATGAGCCCAGGCCATGCCCAGG 3’

P3: 5’ CATCATCACCATCACCATCTCCAGGCGGCAGACACC 3’

P4: 5’
CTATTTTTCGAACTGCGGGTGGCTCCAGCTGCCCCCGGCAGGTCGCACCTTCA
T 3’ or CTAGGCAGGTCGCACCTTCAT (Without streptag)

Notes: CACC (Overhang sequence at 5’ end required for directional TOPO cloning) Signal peptide, Strep-tag, His-tag.

P1= NHP forward primer, P2= His tag antisense primer, P3= His tag NHP ficolin sense primer and P4= Strep-tag NHP ficolin antisense primer

The schematic above shows a representation of colobus and Gorilla ficolin with primers shown; a) colobus ficolin construct nucleotide sequence showing the double tag at both N and C-terminals with primers attached b) Gorilla ficolin construct nucleotide sequence showing the double tag at both N and C-terminals with primers attached.

Note: Both colobus and gorilla ficolin-2 constructs were used independently as either with His-tag or with streptag.

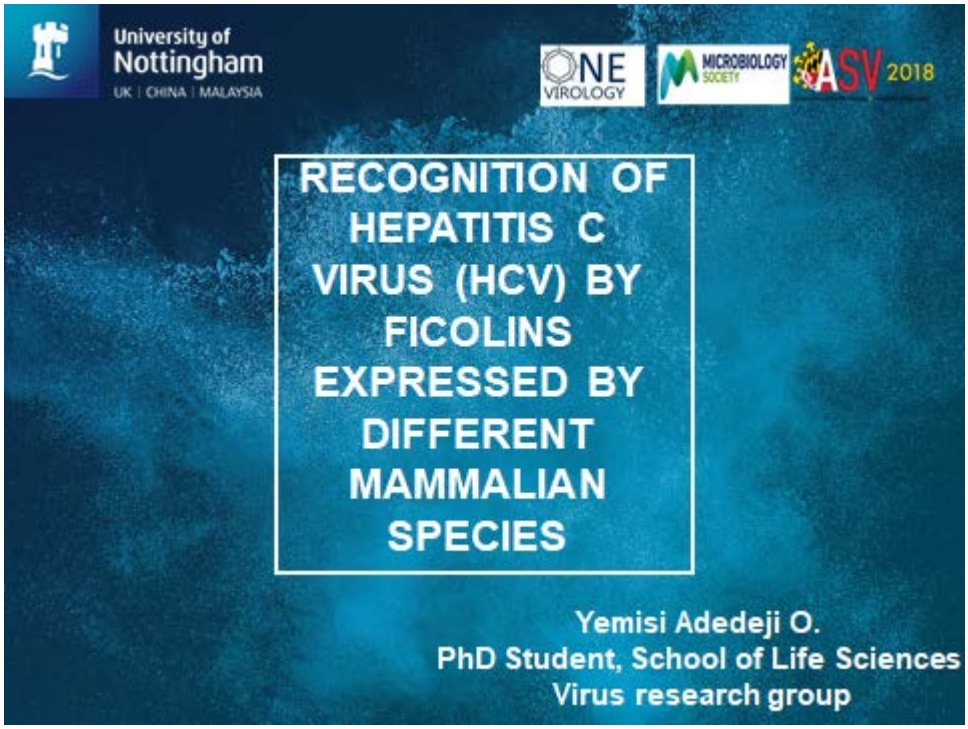
7.2 **Posters and presentations**

7.2.1 Presentation at 37th American Society of Virology Conference

University of Maryland, Maryland United States

Presentation-

“Recognition of Hepatitis C virus (HCV) by ficolins expressed by different mammalian species”



7.2.2 Microbiology Society Annual Conference 2018

RECOGNITION OF HEPATITIS C VIRUS (HCV) BY FICOLINS EXPRESSESD BY DIFFERENT MAMMALIAN SPECIES

University of Nottingham UK | CHINA | MALAYSIA

ONE VIROLOGY

Adedeji O. Yemisi , Christopher P. Mason, Patrick C. McClure, Jonathan K. Ball & Alexander W. Tarr

School of Life Sciences, University of Nottingham, United Kingdom.

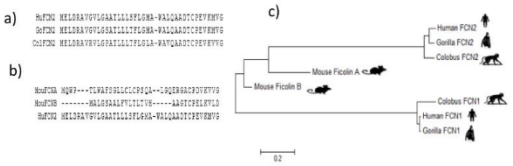
MICROBIOLOGY SOCIETY

Introduction

Ficolins are polymorphic liver expressed pattern recognition receptors (PRRs) that contribute to innate surveillance of virus infections; recognizing carbohydrates such as N- acetyl glucosamine. Three ficolin genes have been identified in humans; *FCN1, FCN2 and FCN3* which encode M-ficolin, L-ficolin and H-ficolin, respectively. Three ficolin-encoding genes have been identified in all non-human primates, while two are found in mice (*fcn-a and fcn- b).* L-ficolin has been demonstrated to bind to HCV virions and inhibit infection. However HCV still persist in the presence of this protein; suggesting that the virus may adapt to avoid recognition of host immune effector. We therefore hypothesized that ficolin from other mammalian species might interact with HCV glycoproteins E1 and E2 and probably neutralize the virus better than L-ficolin. The role of the different species of ficolins might serve as a potential therapeutic anti-viral agent.

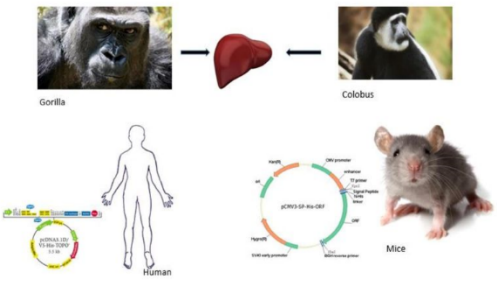
1. Evolutionary relationships of ficolin (*FCN)* genes

- Multiple alignment of the amino acid sequence showed a conservation of 98% for human ficolin 2 (HumFCN2) and non-human primate ficolin 2 (a) while the mouse ficolin (b) showed great variation.
- A Neighbour-joining phylogenetic tree (c) was constructed to understand the evolutionary relationships of ficolin genes from different mammalian species.
- This implies that although ficolin genes from different species might have same structural homology (3c),these proteins are different as well as their recognition pattern of viruses.



2. Cloning of ficolin genes

- To understand the inter-individual molecular relationship of Ficolin genes from different mammalian species, we sequenced their coding region. RNA was extracted from Gorilla and colobus liver and subsequently synthesised into cDNA, amplified and successfully cloned into a mammalian expression vector like the human and mouse ficolins shown below.



3. In-vitro expression of recombinant ficolin from different mammalian species

- Recombinant ficolin protein was expressed from the different mammalian species in human cells and studied by western blotting and silver stain. Proteins at 37kDa (FCNA) and 35kDa (L-ficolin) and higher oligomers were observed.
- The Silver stain confirmed pooled purified dialyzed FCNA, Human ficolin 2 and Gorilla ficolin 2.

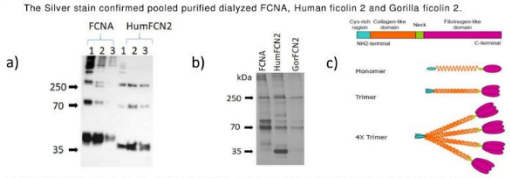
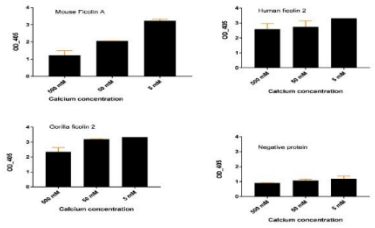


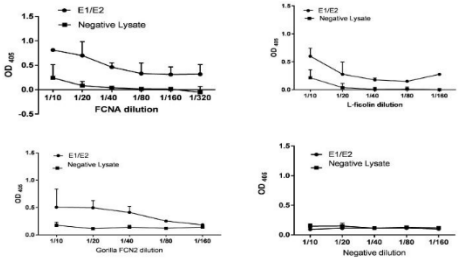
Figure 3; a) FCNA 1, 2, 3 and HumFCN2 1,2,3; represents the harvested protein and two purified eluents respectively. b) silver stain of all purified ficolin c) structural organization of a ficolin.

4. Calcium- dependent binding of Ficolins



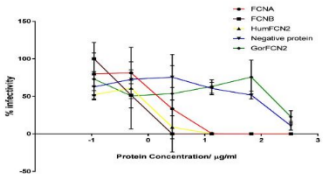
- The binding specificity of the recombinant proteins generated in human cells was an evidence of ficolin binding to different ligands especially N-Acetyl compounds.
- All the proteins showed binding to Acetylated Bovine Serum Albumin (AcBSA) in a calcium dependent manner.

5. Recombinant ficolin of different species interact with HCV Glycoproteins E1 and E2



- HCV genotype 1a (H77) glycoproteins E1 and E2 are highly glycosylated and bind to ficolins from different mammalian species.
- The recombinant ficolin proteins are all bound to E1/E2 heterodimers in a dose dependent manner.

6. Mouse ficolin A and B neutralize HCV more potently than human ficolin 2 and Gorilla ficolin 2



- Neutralization of HCVpp by ficolin from different mammalian species were assessed in a retroviral-based pseudoparticle assay.
- The mouse ficolin (FCNA and FCNB) inhibited the infectivity of HCVpp to different degrees in a dose dependent pattern.

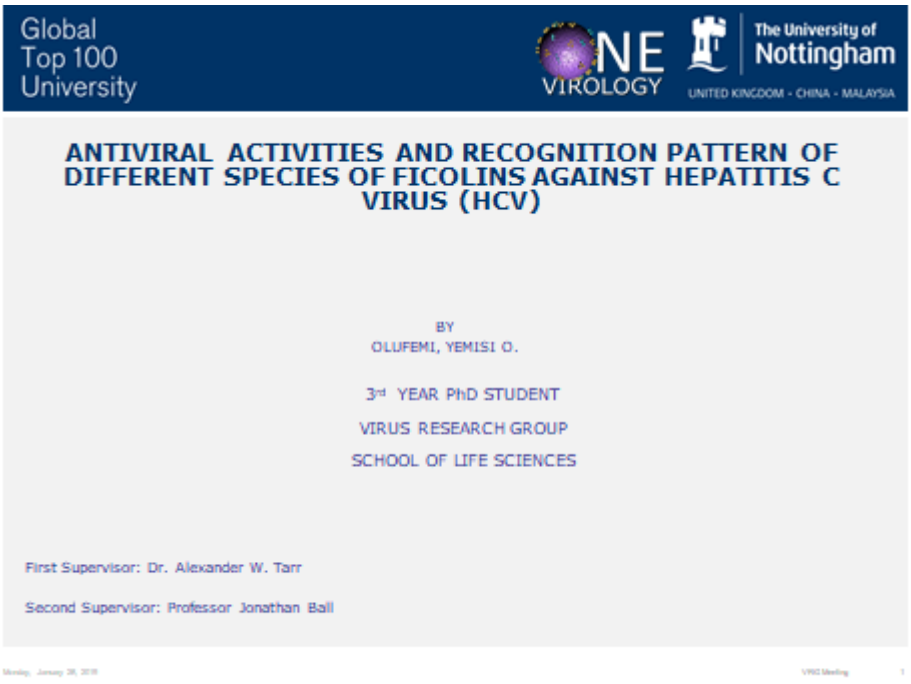
Conclusion

- The binding of ficolin in the different species was specific and in a calcium dependent manner.
- This is the first time the neutralisation of HCV is been assessed by ficolins expressed by different mammalian species and may provide a better insight into how viruses can adapt to different host environments. The role of the these ficolins might serve as a potential therapeutic anti-viral agent.
- Neutralisation of HCV entry by mouse ficolins was more potent than primate ficolin and human ficolin 2 (L-ficolin). The preliminary data of this study support the hypothesis that HCV have evolved in the presence of L-ficolin. Hence, It might be possible to generate novel recombinant chimeric proteins derived from other mammalian species and Human ficolin 2 to serve as new innate immune therapeutic agent.

7.2.3 School of Life Sciences Annual Symposium 2017

Prsentation-

“Antiviral Activities and Recognition pattern of Different species of ficolins against Hepatitis C virus (C)”



7.2.4 Microbiology Society Annual Conference 2017

ANTIVIRAL ACTIVITY OF PRIMATE FICOLINS AGAINST HEPATITIS C VIRUS (HCV) AND OTHER EMERGING VIRAL INFECTIONS

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The University of Nottingham

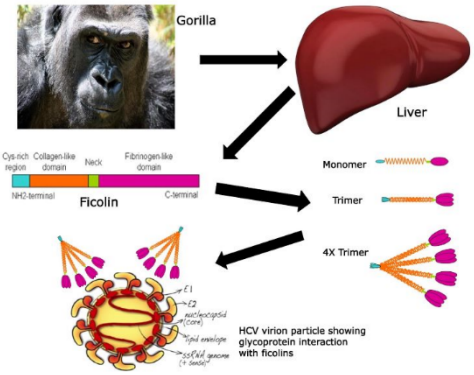
UNITED KINGDOM · CHINA · MALAYSIA

1. Introduction

Hepatitis C virus (HCV) causes chronic liver diseases in 3% of the world's human population[1]. HCV encodes two glycoprotein E1 and E2 which are found on the surface of the viral lipid envelope and they facilitate entry into the host. These glycoproteins have been shown to possess epitopes that are targets of host immune recognition.

Ficolins are liver expressed pattern recognition receptors (PRRs) that contribute to the innate surveillance of virus infections recognising carbohydrates such as N- acetylglucosamine. Three ficolins have been identified in man; M-ficolin (FCN1), L-ficolin (FCN2) and H-ficolin (FCN3) encoded by the *FCN1,FCN2* and *FCN3* ficolin genes respectively. L- ficolin has been demonstrated to bind to HCV virions and inhibit infection[2,3]. However HCV persists in the presence of this protein suggesting that the virus may adapt to avoid recognition of host immune effector.

Three ficolins have also been identified in non- human primates (NHP) like in human while two are found in mice (FCNA and FCNB). However the antiviral activity of non- human primate ficolins is unknown. Thus we sought to assess the antiviral properties of non-human primate ficolins.



HCV virion particle showing glycoprotein interaction with ficolins

ASSESSING ANTIVIRAL ACTIVITY OF PRIMATE FICOLINS

2. Screening of *FCN2* gene in NHP

- To characterise the interactions of NHP *FCN2* gene, liver samples of colobus monkey, marmoset and gorilla were screened.
- Total RNA was isolated, cDNA reverse transcribed from mRNA. Quality of the RNA was confirmed by detecting mRNA expression of glyceraldehyde -3- phosphate dehydrogenase (GAPDH).

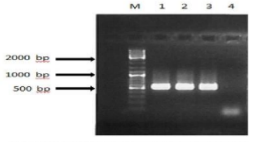


Fig. A. Gel electrophoresis confirmed GAPDH mRNA expression with M as DNA size marker while 1,2 and 3 contained Gorilla, Marmoset and Colobus respectively. Lane 4 is a negative control with no template

Table 1: Designed primers for detection of GAPDH

Primer	Primer Sequence (5'- 3')
NHP_GAPDHF	GAAGGTGAAGGTCGGAGTCA
NHP_GAPDHR	CATGAGTCCCTTCCACGATACC

3. Amplification of *FCN2* gene in NHP Screened

- Specific primers were designed for the detection of NHP *FCN2* genes with L-ficolin as control and further optimised using gradient PCR by which the optimal conditions for the assay were determined.

Table 2. specific primers designed for the amplification of the entire NHP FN2 gene

GC2FCN2_F	ATGGAGCTGGACAGAGCTG
GC2FCN2_R	CTAGGCAGGTCGCACCTTCAT
MAR2FCN2_F	ATGGGACCCGGTCTCCTG
MAR2FCN2_R	CTAGGCGGGCCGCACCTTC

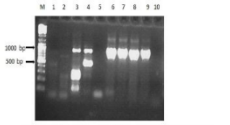


Fig. B: Amplification of the full- length NHP ficolins. M is a DNA marker loaded in the first lane. Lane 1 showed dimers for marmoset. Colobus and Gorilla FCN2 gene at 828bp were detected in lane 3 and 4. The positive L-ficolin plasmid control with same primer set is confirmed from lane 6 to 9.

4. Characterisation of *FCN2* genes in NHP

- Interestingly, the sequenced data of the NHP screened blasted on NCBI showed similarity with NHP *FCN2* gene and human ficolin deposited on Genbank.
- This was aligned by Clutal W using MEGA and a phylogenetic tree was constructed confirming amplified gene when compared with the sequences on GenBank.
- This corresponds with previous analysis performed with a neighbour joining tree which showed a high conservation and variability between NHP *FCN2* genes when compared to others[4].

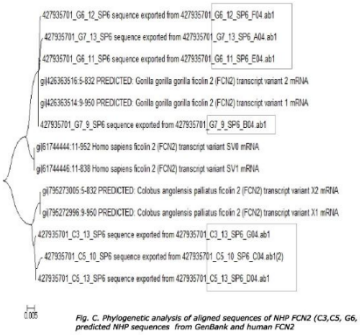


Fig. C. Phylogenetic analysis of aligned sequences of NHP FCN2 (C3,C5, G6, G7) with predicted NHP sequences from GenBank and human FCN2

Future Work

- Production of recombinant NHP FCN2 from different species to show interaction with viruses such as HCV and related hepaciviruses.
- Recombinant FCN2 will be used in binding assays to investigate patterns of binding to their ligands using HCV envelope glycoprotein as target.
- Neutralisation assays will be performed for each cloned ficolin comparing them with the effect of human L- ficolin.
- The ability of NHP FCN2 to neutralise HCV infectivity in the future may be beneficial in terms of novel chimeric molecule with enhanced activity against HCV.

Acknowledgements

We want to acknowledge Twycross Zoo, East Midlands Zoological Society, Atherstone, United Kingdom, for providing the NHP liver samples. Also, Okechukwu Oniawa who is 3rd year PhD student for sorting samples and guiding through the extraction of RNA from these samples.

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