5 Results – The Mechanism of IFITM3's Antiviral Activity

5.1 Introduction

The role of IFITM3 as a broad-acting potent viral restriction factor that is well conserved across many species has been made clear across the preceding two chapters. As discussed in 1.3.1, restriction factors such as tetherin and TRIM5α not only have a physical interaction with HIV-1 particles, but they also trigger a pro-inflammatory response[64,82]. These antiviral proteins recruit TRAF6 leading to the phosphorylation of Tak-1, which stimulates activation of IκB kinase (IKK). This causes the dissociation of IκB and NF-κB, allowing NF-κB to move into the nucleus, resulting in increased expression of NF-κB responsive genes[271]. Tyrosine amino acids Y6 and Y8 in the cytoplasmic domain of tetherin have been shown to be important for recruitment of the TRAF6 signalling complex[82]. Tyrosine Y20 in IFITM3 have been shown to be phosphorylated[111], but no studies thus far have examined if members of the IFITM family also signal in a similar way.

Previous studies have shown that IFITM3 prevents viral particles from exiting the acidic endosome and entering the cytoplasm[4], although the mechanism by which IFITM3 achieves this remains unclear. Several theories to explain this antiviral mechanism have been proposed, including the cholesterol hypothesis and the hemifusion hypothesis. The former of these suggests that IFITM3 interacts with VAPA and disrupts its association with OSBP, which regulates the cholesterol content of endosomal membranes[122]. The authors suggest that an increase in cholesterol may decrease the ability of the viral envelope to fuse with the endosomal membrane through a corresponding decrease in endosomal membrane fluidity. Contrary to this, Desai *et al.* showed that cholesterol-laden endosomes are still permissive to influenza infections[6], so cholesterol upregulation may be a side-effect of IFITM3's action.

The second hypothesis also suggests that IFITM3 prevents complete fusion of the viral and endosomal membranes, but by increasing the positive curvature of the endosomal membrane[115]. This would make it more difficult for a fusion peptide to span the membrane envelopes and trigger fusion. However, IFITM3 has also been shown to restrict a non-enveloped reovirus, which would not need to fuse within the endosome. Furthermore, Li *et al.*[115] used a plasma membrane syncytia-formation

model as a proxy for virus/endosome fusion. Therefore, the authors extrapolated the results of the cell-cell transmission of viruses to cell-viral membrane fusion in the endosome, which may not reflect IFITM3's intracellular activity.

An alternative route to investigate IFITM3's mechanism of action is to look at its binding partners and the roles that they may have in the cell. However, because antibodies specific for IFITM3 only are not available, it is currently necessary to carry experiments out on a tagged protein and to optimise a co-immunoprecipitation assay.

The questions of this chapter are as follows:

i. Does IFITM3 cause the activation of signalling pathways leading to activation of transcription factors that increase expression of IFN or other pro-inflammatory cytokines in a similar manner to other restriction factors?
ii. Can a robust co-immunoprecipitation assay be developed to pull down interacting partners of HA-tagged IFITM3?

5.2 Does Over-expression of IFITMs Cause an Increase in Intracellular Signalling?

Given that IFITM3's antiviral effect occurs early in the virus life cycle, we hypothesised that it might also trigger a pro-inflammatory response in a similar way to tetherin and TRIM5α[64,82], via signalling by transcription factors such as NF-κB. Several groups have tested the signalling capacity of restriction factors by using a dual-luciferase system developed by Jeremy Luban's group[64]. Each well of cells is transfected with three plasmids: a transfection control plasmid (expressing Renilla luciferase [luc]), a Firefly luc reporter plasmid containing binding sites for a transcription factor (*e.g.* NF-κB), and an ISG expression plasmid. If the ISG signals via NF-κB, induction of reporter gene expression will occur.

In these experiments three reporter constructs have been used; the NF-κB reporter construct has several κB binding sites that NF-κB can bind to after dissociating from IκB. The second contains an ISRE to which the ISGF3 complex can bind and the third contains an IFNβ promoter to which NF-κB, AP-1, ATF-2, IRF3 and other proteins bind to as part of the 'enhanceasome'[272]. These constructs therefore reflect several different signalling pathways.

To test whether or not IFITM3 signals in a similar way to other ISGs, HEK293 cells were transfected with the transfection control plasmid, the Firefly luc reporter plasmid (controlled by NF-κB binding domain or an IFNβ promoter) and an expression plasmid expressing human IFITM3, the mitochondrial antiviral signalling (MAVS), tetherin, or a mutant tetherin (Y6.8A). Tetherin and MAVS have both been shown to be strong inducers of NF-κB and IRF3[273], and are therefore used as positive controls for this system. The Y6.8A mutagenised form of tetherin has been shown to have reduced signalling activity via NF-κB[82].

In the 293 cells, only MAVS expression induced activity of the NF-κB and IFNβ promoter constructs by 231- and 737-fold, respectively, compared to an empty vector control (Figure 56). Tetherin expression resulted in less promoter activity from these constructs (64- and 2.9-fold) consistent with published data[64,82]. Mutant tetherin had 4-fold less activity than the wildtype protein for NF-κB signalling. In all cases IFITM3 activity was less than tetherin Y6.8A (Figure 56).

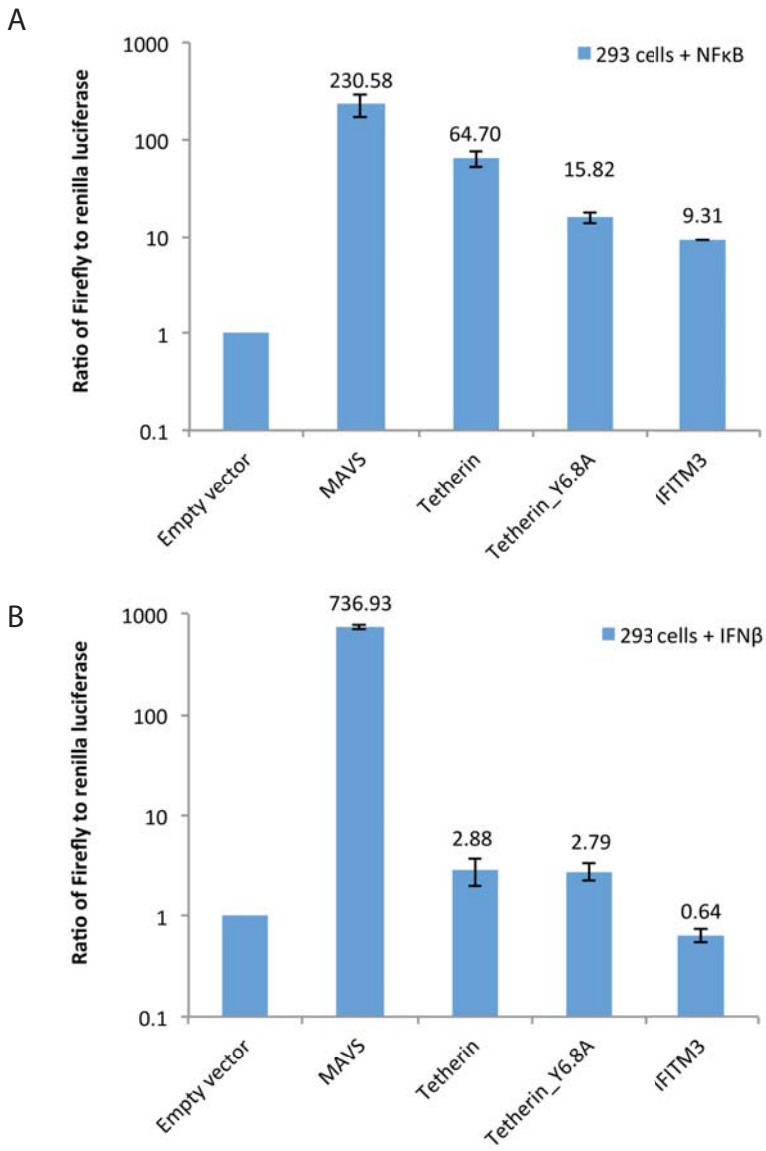


Figure 56: Signalling via NF-κB and an IFNβ promoter is not induced by expression of human IFITM3 in HEK293 cells

HEK293 cells were transfected with a Firefly luciferase reporter plasmid under the control of a κB binding site (A) or an IFNβ promoter (B). Cells were co-transfected with a transfection control plasmid, and a gene expression plasmid. Media was changed 24 h post-transfection and Firefly luciferase activity, in relation to Renilla luciferase activity, was measured 24 h later. Ratios were normalised to transfection with an empty vector control. Error bars are standard deviation of the mean, n=3.

To test if cell infection causes an enhancement in cell signalling by IFITM3, influenza A was used to infect HEK293 cells 24 hours post-transfection (Figure 57). Infection had no effect on the relative signalling activity of MAVS, tetherin and IFITM3.

In addition, the effect of various agonists for endosomally-located TLRs on cell signalling by IFITM1, 2 and 3 were tested. TLRs are known to detect specific pathogen components and initiate NF-κB signalling. CpGs and poly I:C are ligands for TLR9 and TLR3 respectively[274]. HEK293-T cells were transfected with the transfection control plasmid, the Firefly luc reporter plasmid (controlled by a κB binding site or an ISRE) and an expression plasmid expressing either MAVS, TRIM5α, human IFITM1, IFITM2, IFITM3, or an empty vector.

Expression of IFITM1, 2, or 3 did not upregulate signalling significantly more than transfection of an empty vector control. Addition of CpGs or poly I:C after transfection of the IFITM proteins had no impact on signalling via κB binding sites (Figure 58A) or an ISRE (Figure 59), respectively. Expression of IFITM2 with addition of poly I:C resulted in signalling via NF-κB to be 1.5-fold higher than without stimulation (Figure 58B), but the raw values for this experiment were very low and this result did not reach significance. These experiments were repeated in A549 cells however they were also difficult to transfect, which made the data very unreliable (data not shown).

To test whether or not signalling would increase if IFITM3 were constitutively expressed in the cell, a cell line stably expressing human IFITM3 (293T_IFITM3) was made. These cells were also seeded and transfected with a Renilla control plasmid and a reporter plasmid only or an additional empty vector control. As in the previous experiments, cells were subsequently mock-infected (Figure 60A), or infected with influenza A (Figure 60B). Constitutive expression of IFITM3 did not promote signalling via either NF-κB or an IFNβ promoter, regardless of infection.

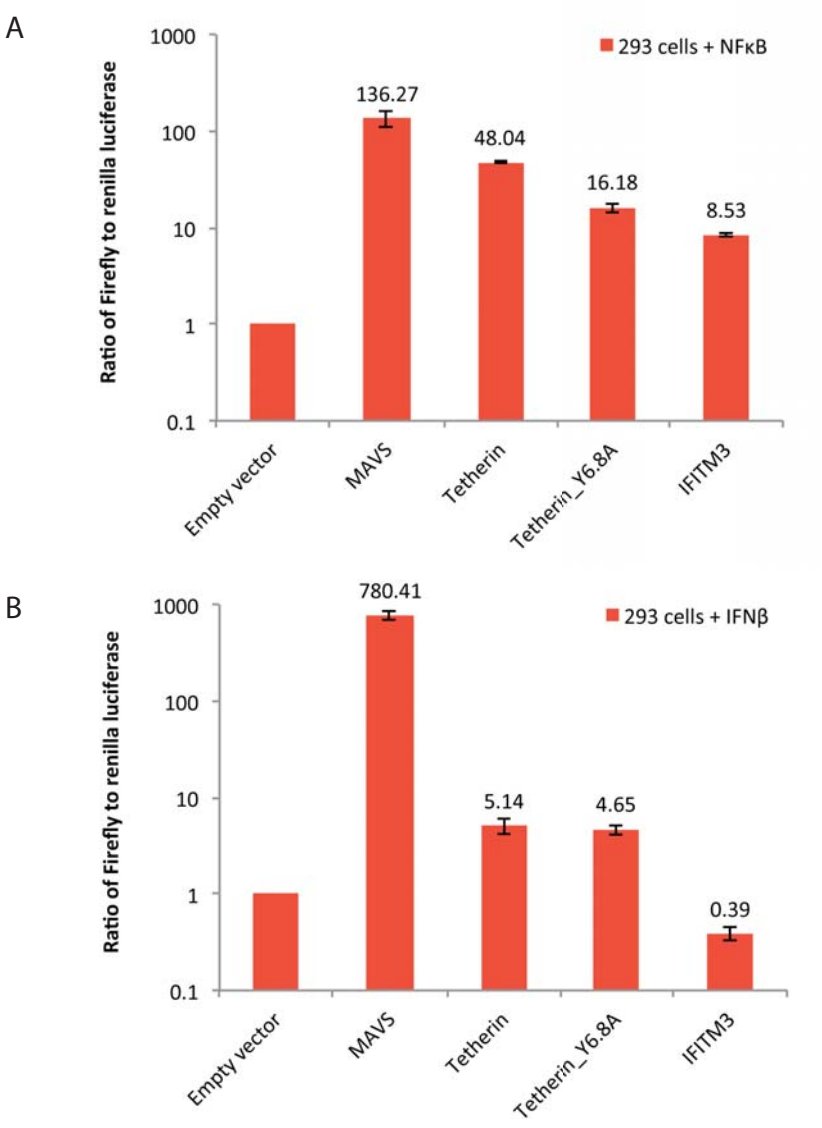


Figure 57: Signalling via NF-κB and an IFNβ promoter is not induced by expression of human IFITM3 prior to an influenza A infection in HEK293 cells

HEK293 cells were transfected with a Firefly luciferase reporter plasmid under the control of a κB binding site (A) or an IFNβ promoter (B). Cells were co-transfected with a transfection control plasmid, and a gene expression plasmid. Cells were stimulated 24 h post-transfection with influenza A/WSN/33 at an MOI of 1. Firefly luciferase activity, in relation to Renilla luciferase activity, was measured 24 h post infection. Ratios were normalised to transfection with an empty vector control. Error bars are standard deviation of the mean, n=3.

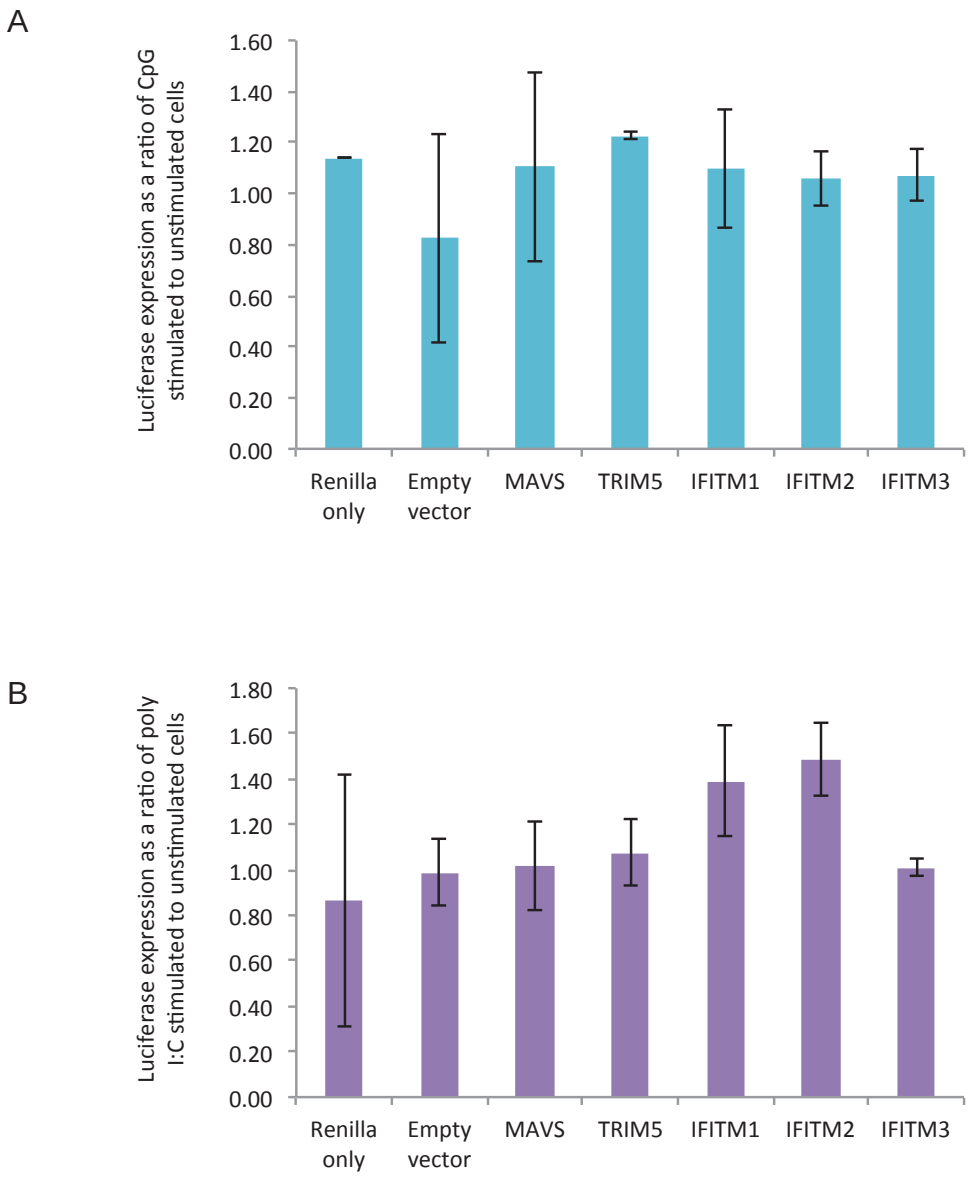


Figure 58: Addition of CpGs or poly I:C does not increase signalling via NF-κB after expression of human IFITM1, 2, or 3 in HEK293-T cells

HEK293-T cells were transfected with a Firefly luciferase reporter plasmid under the control of a κB binding site. Cells were co-transfected with a transfection control plasmid, and a gene expression plasmid. Cells were stimulated with CpGs (A) or poly I:C (B) and compared to unstimulated cells. Firefly luciferase activity relative to Renilla luciferase activity was measured 24 h post-transfection and is given as a ratio of stimulated to unstimulated cells. Error bars are standard deviation of the mean, n=3.

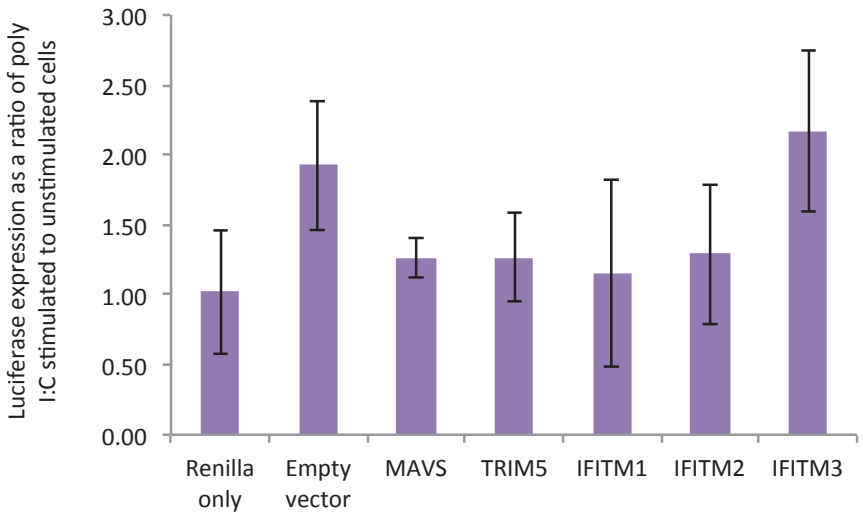
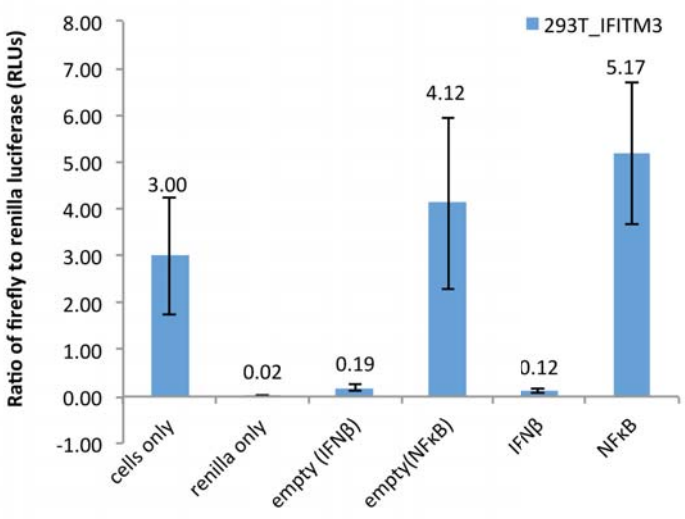


Figure 59: Addition of poly I:C does not increase signalling via an ISRE after expression of human IFITM1, 2, or 3 in HEK293-T cells

HEK293-T cells were transfected with a Firefly luciferase reporter plasmid under the control of an ISRE. Cells were co-transfected with a transfection control plasmid (Renilla), and an ISG expression plasmid. Cells were co-stimulated with poly I:C and compared to unstimulated cells. Firefly luciferase activity in relation to Renilla luciferase activity was measured 24 h post-transfection and is given as a ratio of stimulated to unstimulated cells. Error bars are standard deviation of the mean, n=3.

A



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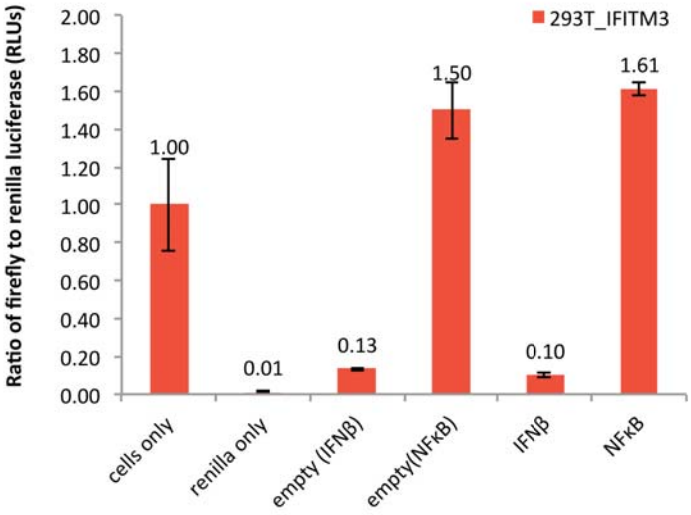

Figure 60: Signalling via NF-κB and an IFNβ promoter is not induced in HEK293-T cells constitutively expressing of human IFITM3

HEK293-T cells constitutively over-expressing human IFITM3 were transfected with a Firefly luciferase reporter plasmid under the control of κB binding site or an IFNβ promoter and a transfection control plasmid. 24 h post-transfection cells were mock infected (A) or infected with influenza A/WSN/33 at an MOI of 1 (B). Firefly luciferase activity, relative to Renilla luciferase activity, was measured 24 h later. An empty vector was used as a control. Error bars are standard deviation of the mean, n=3.

5.3 Optimisation of Co-immunoprecipitation Protocols for Human IFITM3

To characterise what proteins IFITM3 interacts with, robust methods for co-immunoprecipitation (co-IP) of IFITM3 are required. Here such co-IPs were developed. This procedure utilised a non-denaturing detergent to lyse the cells under conditions designed to leave protein-protein interactions intact, such that IP of IFITM3 would potentially co-IP interacting proteins. Two methods of co-IP were used in this project – the first used magnetic beads coated in Protein A, which allowed easy binding of the anti-HA antibody and washing, and the second (for large-scale preps) utilised agarose beads pre-bound with an anti-HA antibody.

5.3.1 Using Magnetic Beads to Precipitate IFITM3

IFITM3 shares 90 % sequence similarity with IFITM2, which means that antibodies specific for IFITM3 only are not available. Therefore for the following experiments we have expressed a C-terminally HA-tagged IFITM3 protein in A549 cells (low in IFITM3 expression) to allow specific detection and IP by anti-HA antibodies. A549s cells were harvested using a non-denaturing lysis buffer and the total cellular protein extracted. A co-IP was carried out using the magnetic Dynabeads® Protein A Immunoprecipitation Kit onto which the anti-HA antibody was attached. To prevent the antibody dissociating from the beads during the elution step (the heavy and light chains would mask many proteins during some analyses such as mass spectrometry) two different elution solutions were tested along with two forms of cross-linking the antibody to the beads.

Using the standard protocol (Protein A affinity binding of antibody to magnetic beads without cross-linking and glycine elution), two large bands at 25 kDa and 55 kDa were detected by Coomassie (Figure 61A, lane 1), which indicated the presence of the light and heavy chains of the antibody. The target protein (IFITM3) was detected by Western blot (Figure 61B, lane 1), indicating that the IP was successful, but IFITM3 was not detected on the Coomassie gel. As the Coomassie blue stain is much less sensitive than the Western blot, this suggested that the antibody dissociating from the beads could interfere with any downstream analysis as it was at a much greater abundance than IFITM3. To circumvent this problem, competitive elution using HA peptide was tested to prevent elution of the antibody from the beads but allow elution of the HA-tagged bait protein. However IFITM3 was not detected in the eluent by Western blot (Figure 61B, lane 2).

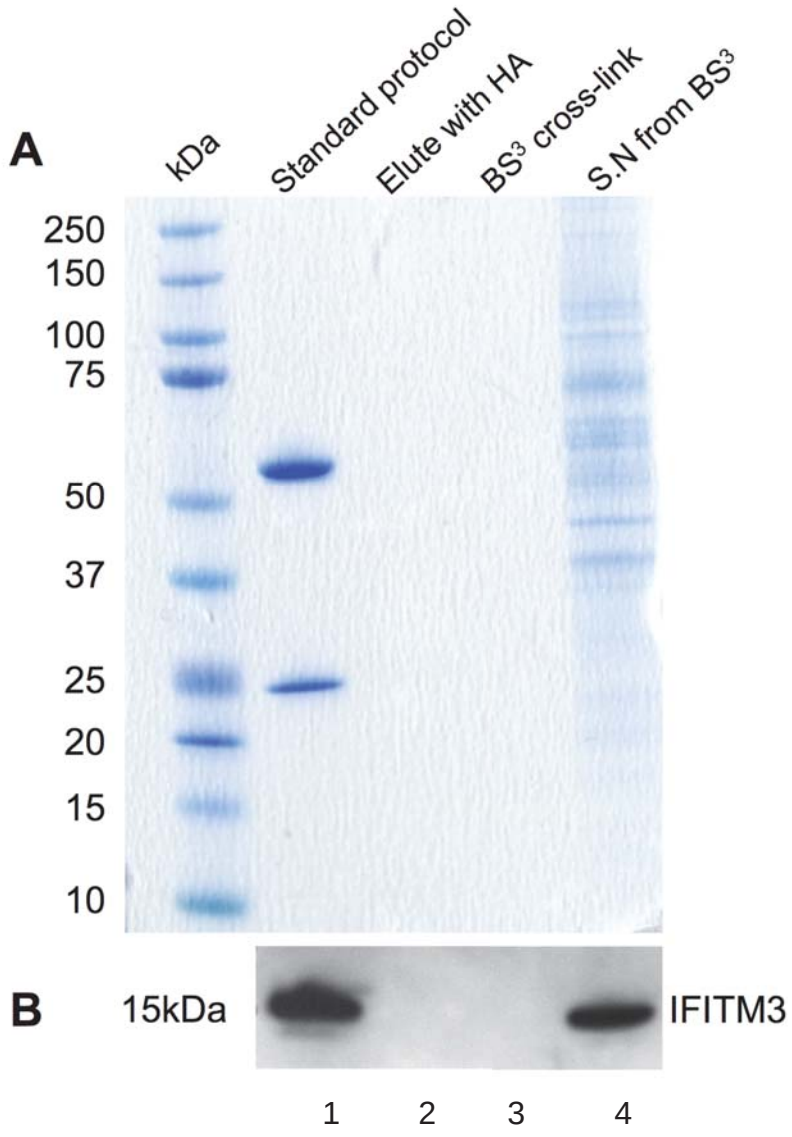

Figure 61: BS^3 cross-linking prevents efficient elution of IFITM3-HA

Immunoprecipitation was carried out using the standard protocol (Protein A affinity binding of antibody to beads without cross-linking and glycine elution), HA elution (Protein A affinity binding of antibody to beads without cross-linking and HA peptide elution) or BS^3 cross-linking with glycine elution. Eluates and supernatants from the cross-linking were analysed by Coomassie (A) and Western blot using the anti-HA antibody (B). S.N; supernatant.

A second approach using an irreversible water-soluble conjugate cross-linker, Bis(sulfosuccinimidyl)suberate (BS^3), was carried out to bind the fragment crystallisable (Fc) region of the antibody to the Protein A component of the magnetic bead. When compared with the standard protocol, cross-linking with BS^3 reduced the amount of antibody that dissociated during elution (Figure 61A, lane 3). However, IFITM3 could no longer be detected in the elution fragment by Western blot (Figure 61B, lane 3), but was still detected in the washes after antigen binding (Figure 61B, lane 4). This implies that after cross-linking the beads with BS^3, IFITM3 could no longer bind to the anti-HA antibody. An alternative method of cross-linking using dimethyl pimelimidate (DMP) to permanently bind the antibody to the magnetic bead was tested, but also proved disruptive to the Fab region of the HA antibody so that all of the target protein was in the supernatant and not in the eluent (data not shown).

The concentration of HA peptide was increased by 5-fold to increase the likelihood that IFITM3 would be eluted from the beads (Figure 62). Cross-linking by BS^3 before elution with either glycine or HA peptide resulted in no detection of IFITM3 by Western blot in the eluate (Figure 62B, lanes 2 and 4). HA elution without cross-linking (Figure 62B, lane 5) allowed detection of IFITM3 in the eluate, but faint bands corresponding to the heavy and light chains of the antibody were detected by Western blot (data not shown). However these bands were not detected by the less sensitive Coomassie assay (Figure 62A, lane 5) unlike for the non-cross-linked glycine elution (Figure 62A, lane 3). Therefore elution using a high concentration of HA peptide without cross-linking was an effective method of eluting IFITM3-HA from the magnetic beads.

In order to submit co-immunoprecipitation samples for methods such as mass spectrometry, at least 10 mg of starting material must be bound to the beads to allow elution of enough protein for analysis. Upon scaling-up the experiments from 1 mg to 10 mg, the magnetic beads clumped and aggregated in the tube, preventing efficient washing or elution. Increasing the volume of beads decreased the efficiency of binding the anti-HA antibody to the bead (data not shown). To circumvent this problem, agarose beads that were pre-bound with an anti-HA antibody were purchased. Although elution from agarose beads can be less efficient than from magnetic beads, this avoided the difficulties of optimising the antibody-binding conditions for the magnetic beads.

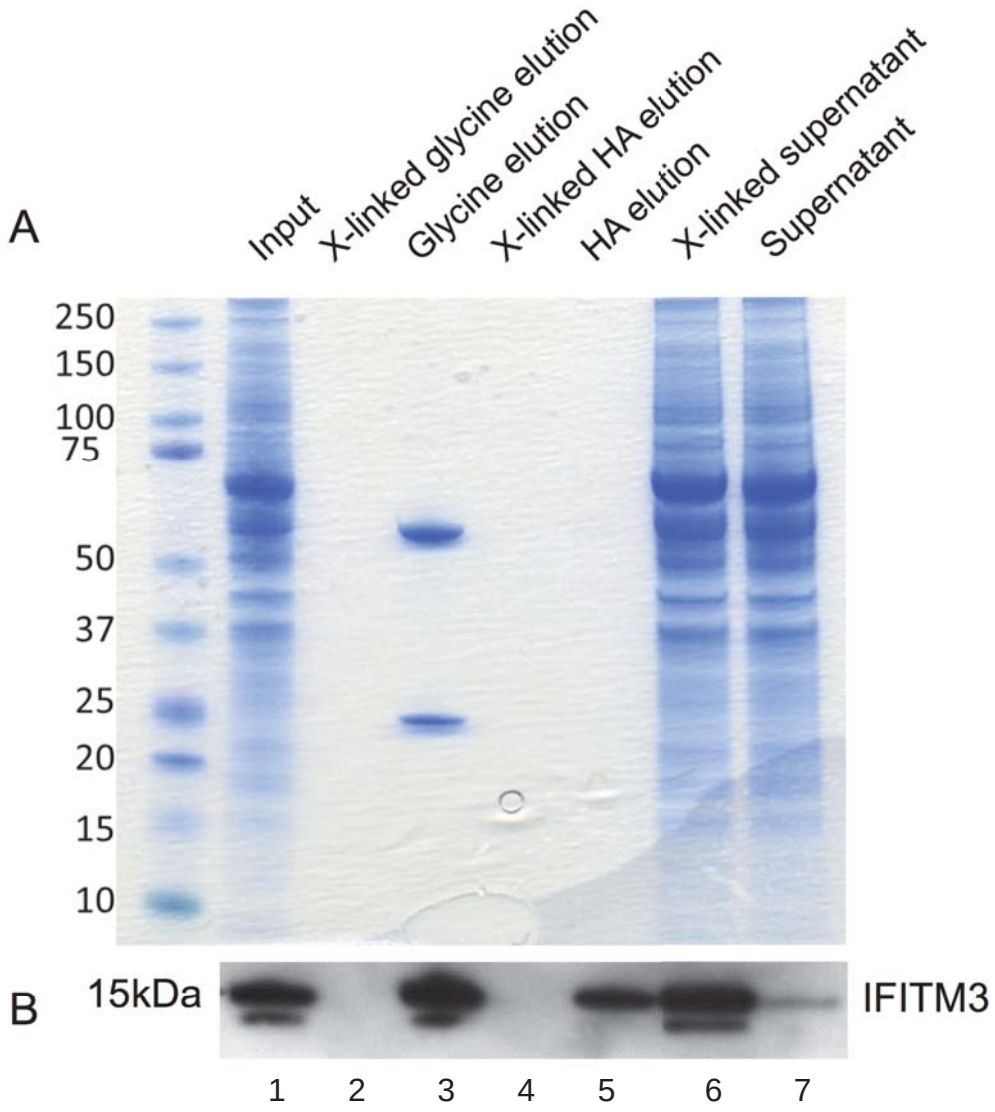

Figure 62: Competitive elution of IFITM3-HA using HA peptide is more effective than glycine elution

Co-immunoprecipitation was carried out using cross-linking (X-linked) by BS^3, or the standard protocol eluting with either glycine or HA peptide. Total cell lysate before IP (Input), Cross-linked supernatants from the glycine elution (x-linked supernatant), supernatants from the non-crossed-linked glycine elution (Supernatant) and eluates from both conditions were analysed by Coomassie (A) and Western blot using the anti-HA antibody (B).

5.3.2 Using Agarose Beads to Precipitate IFITM3

A549 cells over-expressing IFITM3_HA were lysed and the protein supernatant bound to 100 µl of anti-HA-bound agarose beads. The protein solution was incubated on the beads for 3 h at 4 °C. The beads were washed and competitive elution carried out using HA peptide, as previously. The eluate was concentrated using a centrifugal concentrator with a 5 kDa molecular weight cut-off membrane.

IFITM3 was not detected in the eluate by Coomassie (Figure 63A, lane 5), but the wash steps showed that non-bound proteins were removed from the agarose beads. A Western blot using the anti-HA antibody shows that there is still a large amount of IFITM3 in the supernatant post-agarose binding (Figure 63B, lane 2), suggesting that the beads were saturated or the binding had not gone to completion. A small amount of IFITM3 was detected in wash 1 (Figure 63B, lane 3), but far less in wash 2. Importantly, IFITM3 was successfully eluted by HA after wash 2 (Figure 63B, lane 5), and was not detected in the filtrate after using the centrifugal concentrator (Figure 63B, lane 7).

To test if the IP conditions allow the co-IP of IFITM3 interacting proteins we determined the co-IP of IFITM3 with VAPA[122], previously shown to interact with IFITM3 (Figure 63C). VAPA (33 kDa) was present in the input protein and the post-agarose binding supernatant (lanes 1 and 2), but was not present in any of the washes or elutions.

The co-IP was repeated using 50 % more beads and the incubation during rotation was increased by 2 h. Again, IFITM3 could be clearly seen in wash 1 (Figure 64B, lane 3) and in the elution (Figure 64B, lane 5), but now VAPA could also be detected in wash 1 (Figure 64B, lane 3) and faintly detected in the elution (Figure 64C, lane 5).

To determine if IFITM3 was detected in the elution simply because it was so heavily over-expressed in the cells and binding non-specifically to the beads, a further control using an anti-myc antibody to IP was carried out (Figure 65). Supernatant from IFITM3_HA cells was bound to agarose beads attached to either an anti-HA antibody or an anti-myc antibody. A small but detectable amount of IFITM3 did bind non-specifically to the anti-myc beads, but was removed by the

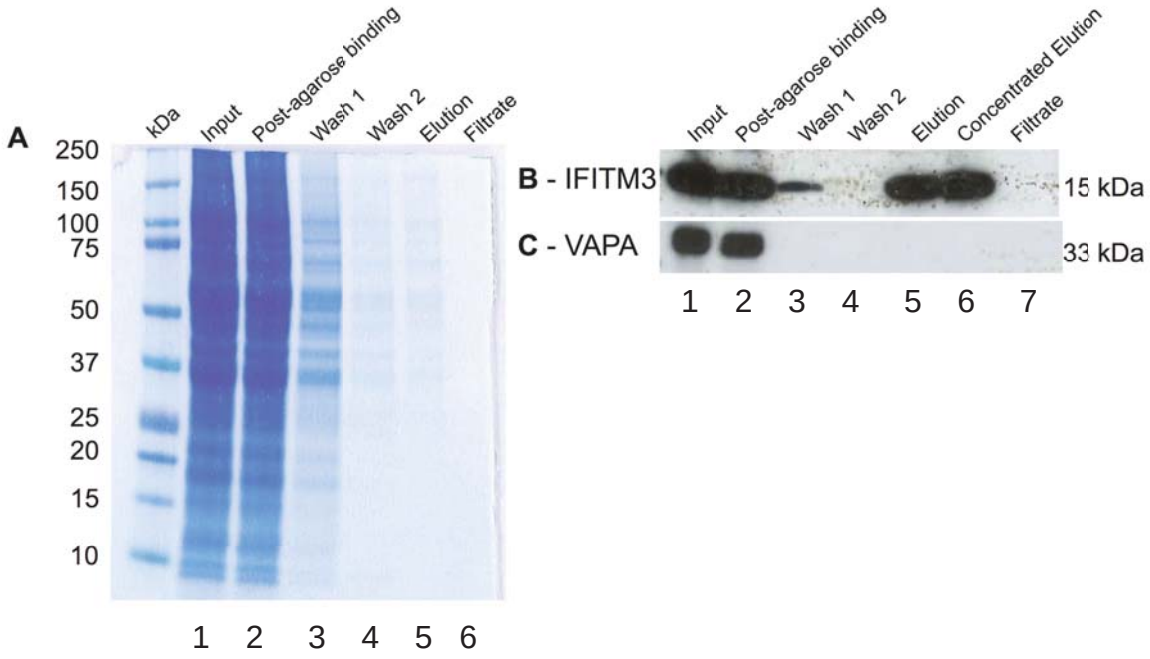

Figure 63: Immunoprecipitation of IFITM3-HA from agarose beads bound to an anti-HA antibody

3 mg of protein from A549-huIFITM3_HA cells was washed over 100 µl of agarose beads bound with an anti-HA antibody, for 3 h at 4 °C. Supernatants were collected from each wash step and elution was performed using HA peptide. An aliquot of each supernatant was run on an SDS-PAGE gel and all proteins detected by Coomassie (A). Western blots were carried out to specifically detect IFITM3 (B) and VAPA (C).

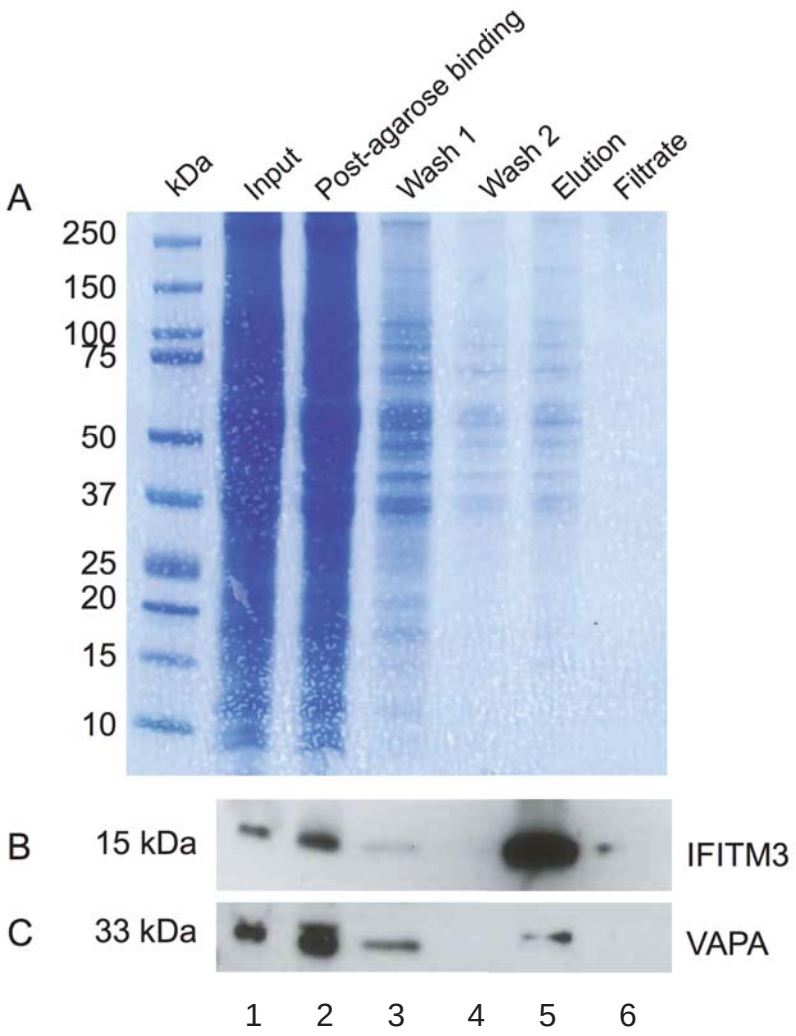

Figure 64: VAPA co-immunoprecipitates with IFITM3-HA

3 mg of protein from A549-huIFITM3_HA cells was washed over 150 µl of agarose beads bound with an anti-HA antibody, for 5 h at 4 °C. Supernatants were collected from each wash step and elution with HA peptide. An aliquot of each supernatant was run on an SDS-PAGE gel and all proteins detected by Coomassie (A). Western blots were carried out to specifically detect IFITM3 (B) and VAPA (C).

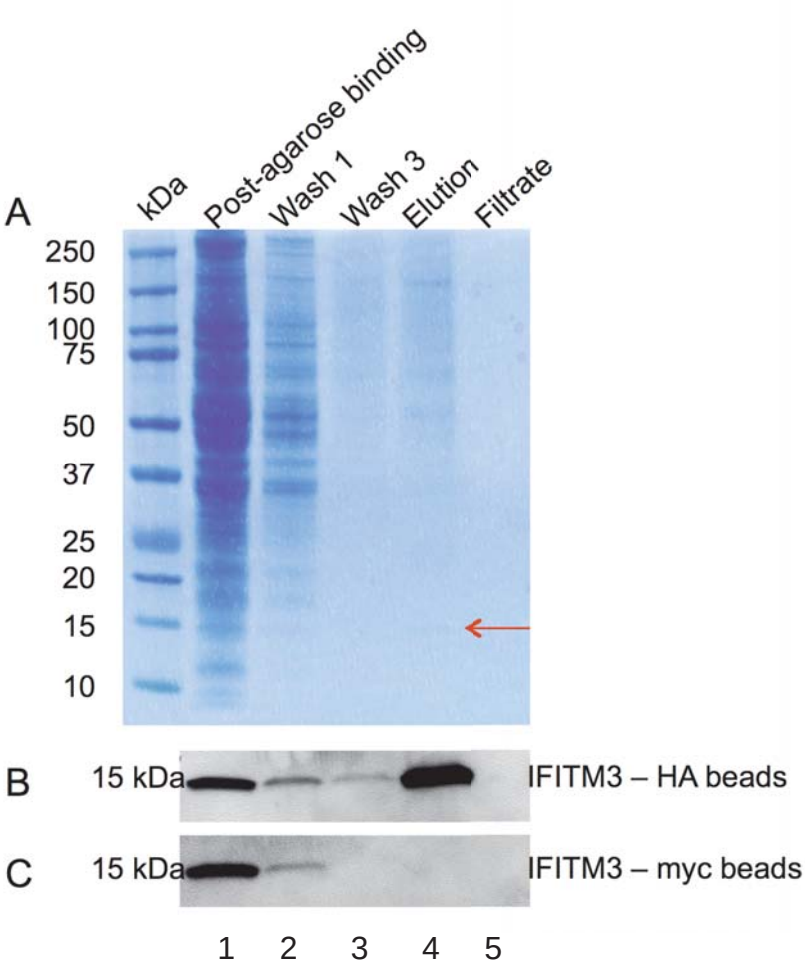

Figure 65: IFITM3-HA does not immunoprecipitate from agarose beads bound to an anti-myc antibody

5 mg of protein from A549-huIFITM3_HA cells was washed over two aliquots of 150 µl of agarose beads bound with an anti-HA antibody or an anti-myc antibody, for 5 h at 4 °C. Supernatants were collected from each wash step and eluted with HA peptide. An aliquot of supernatants from the anti-HA beads was run on an SDS-PAGE gel and all proteins detected by Coomassie (A). Red arrow indicates IFITM3. Western blots were carried out to specifically detect IFITM3 precipitated using anti-HA (B) and anti-myc (C) beads.

washing (Figure 65C, lane 2-3) and IFITM3 was not detected in the eluate (Figure 65C, lane 4). IFITM3 was successfully eluted from the anti-HA beads (Figure 65B, lane 4). Proteins in the supernatants from the anti-HA beads were detected by Coomassie staining (Figure 65A) and IFITM3 was identified in the eluate (Figure 65A, lane 4).

In conclusion, an efficient co-IP protocol was developed using agarose beads pre-attached to an anti-HA antibody, and competitive elution was carried out using HA peptide. The known interaction between IFITM3 and VAPA was confirmed, but the amount of VAPA detected was very low.

5.4 Discussion of Results

5.4.1 Signalling

The role of IFITM3 as an anti-viral molecule that prevents virus release into the cytoplasm has been well established. However, the cellular location and transmembrane structure of IFITM3 made it a candidate for an additional immune signalling function, triggering the expression of proinflammatory genes. Thus far, a signalling role has been established for nearly half of the 75 distinct members of the E3-ligase TRIM family of proteins[275]. Furthermore, tetherin has also been shown to induce a NF-κB pro-inflammatory response[82].

Firefly luciferase reporter plasmids controlled by κB binding domains, an ISRE or an IFNβ promoter were used to establish whether or not IFITM proteins could signal via different transcription factors with or without secondary stimulation. Although positive control proteins MAVS and tetherin could clearly initiate NF-κB signalling, signalling was not detected after expression of any of the *IFITM* genes. The possibility that IFITM proteins require secondary activation to signal, such as TLR stimulation or influenza infection, was also investigated, but no signalling was detected. It nevertheless remains possible that the IFITM proteins signal via a pathway and a transcription factor that was not tested here.

After influenza A infection NF-κB expression was reduced compared to uninfected cells. It is likely that this occurred because of NS1 supressing the NF-κB response[276,277]. NS-1 has a dsRNA binding domain that sequesters the influenza genome and prevents its recognition by other innate immune proteins such as PKR[276], a kinase known to phosphorylate IκB and initiate NF-κB signalling.

Cell-type dependent differences were detected between different assays. Here HEK293-T cells had a poor transfection efficiency, resulting in low raw RLUs for all samples. In addition the empty vector controls resulted in activation of NF-κB, making any small effect of IFITM proteins impossible to detect. This could be due to endotoxin contamination in the plasmid preparation causing TLR stimulation.

Furthermore, some evidence suggests that HEK293-T cells, unlike HEK293 cells, do not express TLR3[278]. If stimulation of the TLR is necessary for IFITM signalling, it would not be detected in these cells. However, some infection assays have been

performed in HEK293-T cells and shown that IFITM3 can restrict Marburg virus, Ebola virus, and IAV *in vitro*[2], suggesting that TLR3 is unimportant for primary restriction. Signalling by tetherin in HEK293-T cells was significantly reduced compared to in HEK293 cells (data not shown). Since spleen tyrosine kinase (Syk) is essential for signalling by tetherin[279] it is possible that HEK293-T cells do not express this protein, and it is not known if Syk is necessary for IFITM3 signalling.

HEK293 cells were also used here and transfected with plasmids encoding binding sites for NF-κB or an IFNβ promoter[271] and a Firefly luciferase reporter along with human IFITM3. The RLUs for positive controls were much higher in these cells and so more reliable, however no signalling by IFITM3 via the IFNβ promoter was detected. IFITM3 stimulated 25-fold less and 7-fold less signalling via NF-κB than MAVS or tetherin respectively and half as much signalling as the mutant tetherin Y6.8A known to be defective for signalling. Furthermore these experiments were repeated in HEK293-T cells constitutively expressing IFITM3 to differentiate between potential signalling in cells in which IFITM3 is upregulated after IFN stimulation and those constitutively expressing high levels of protein, such as HepG2 cells[280]. However no signalling via NF-κB was detected in this system either. Together, these data suggest that IFITM3 does not signal via the innate signalling system at a biologically meaningful level.

5.4.2 Protein-protein Interactions Involving IFITM3

In order to better understand the mechanism by which IFITM3 confers anti-viral resistance, a co-immunoprecipitation to identify binding partners was performed resulting in conditions where HA peptide competitive elution of IFITM3 from commercial HA-coupled agarose beads was an effective method of immune precipitating IFITM3.

VAPA has been identified as a specific interaction partner for IFITM3 using a yeast 2-hybrid technique[122]. We also showed VAPA co-immunoprecipitated with IFITM3 by Western blot. However the band is quite faint compared to the previous study and when detected was also eluted in early washing steps. These observations could be due to differences in the experimental procedures between this study and that carried out by Amini-Bavil-Olyaee *et al.*,[122] that identified VAPA as an interacting protein. Alternatively, VAPA interaction may be transient and weak leading to the difference observed here.