2 Materials and Methods

2.1 Primers Used in this Study

Table 3: Primers used in this study

NUMBER	NAME	SEQUENCE 5' - 3'
1	IFITM3 F2	TGAGGGTTATGGGAGACGGGGT
2	IFITM3 R2	TGCTCACGGCAGGAGGCCCGA
3	SES003_F	GCTTTGGGGGAACGGTTGTG
4	SHORTER IFITM3 R2	TGCTCACGGCAGGAGGCC
5	EXON1 R1	CTTTAGGAGAGGGAGGAAAGA
6	EXON1 F2	CACTAACAAGATGAGCCTTG
7	EXON1 R2	GAACAGGGACCAGACGACAT
8	EXON1 F3	TCTTCGCTGGACACCATGAA
9	EXON1 R3	GAACTGCTCTGGGCTAGTGG
10	INTRON F2	ACTTGTGTGTCCCTGTGACTG
11	INTRON R1	ATGAGGATGCCCAGAATCAG
12	EXON2 F1	CTATGCCTCCACCGCCAAGTG
13	INTRON F3	AGCCAATGAGGAGACGGAG
14	SFFV_F	TGCTTCTCGCTTCTGTTCG
15	WPRE_R	CCACATAGCGTAAAAGGAG
16	M13F	GTTTTCCCAGTCACGAC
17	M13R	CAAGGAAACAGCTATGAC
18	ALT SPLICE 5'UTR IFITM3	GCCCGGCAGAGTGGCCAG
19	2ND ALT SPLICE 5'UTR IFITM3	AAAGTGCTGGGATTACAGGCG
20	ALT_SPLICE2_IFITM3_F’	GACCCCAGAGTCCAGTCTGAG
21	CMV FORWARD	CGCAAATGGGCGGTAGGCGTG
22	TK POLYA REVERSE	CTTCCGTGTTTCAGTTAGC
23	F'HUMAN_IFITM3_NONCODONOP	ACTGTCCAAACCTTCTTCTCTC
24	R'HUMAN_IFITM3_NONCODONOP	AGCACAGCCACCTCGTGCTC
25	F'HUMAN_IFITM2_NONCODONOP	ATTGTGCAAACCTTCTCTCCTG
26	R'HUMAN_IFITM2_NONCODONOP	ACCCCCAGCATAGCCACTTCC

27	F'HUMAN_IFITM1_NONCODONOP	AGCACCATCCTTCCAAGGTCC
28	R'HUMAN_IFITM1_NONCODONOP	TAACAGGATGAATCCAATGGTC
29	CHIFITM3_F’	GGAGTCCCACCGTATGAAC
30	CHIFITM3_R’	GGCGTCTCCACCGTCACCA
31	CHIFITM2_F’	AGGTGAGCATCCCGCTGCAC
32	CHIFITM2_R’	ACCGCCGAGCACCTTCCAGG
33	CHIFITM1_F’	AGCACACCAGCATCAACATGC
34	CHIFITM1_R’	CTACGAAGTCCTTGGCGATGA
35	CHGAPDH_F'	ACTGTCAAGGCTGAGAACGG
36	CHGAPDH_R'	GCTGAGGGAGCTGAGATGA
37	GAPDH_MRNA_F'	GGCTGAGAACGGGAAGCTT
38	GAPDH_MRNA_R'	AGGGATCTCGCTCCTGGAA
39	HIFITM3_UNIQUE_F'	TGGACACCATGAATCACACTGTC
40	HIFITM3_UNIQUE_R'	GAGCATTCCCTGGGGCCATA
41	HIFITM3_UNIQUE_T7_F'	TAATACGACTCACTATAGGGTGGA CACCATGAATCACACTGTC

2.2 General Molecular Biology Techniques

2.2.1 PCR

The Polymerase Chain Reaction (PCR) was used to amplify DNA using thermal cycling (T MJ Research PT C-223 peltierthermal cycler). Unless otherwise stated, the following thermocycling conditions were used: 98 °C for 30 seconds (s); 29 cycles of (98 °C, 10 s; 61 °C, 30 s; 72 °C, 1 minute [min]); 72 °C for 5 min. Each reaction contained 100 ng of template cDNA the components of the PCR mastermix (Table 4, Finnzyme) in a reaction volume of 50 µl.

Table 4: PCR mastermix

Reagent	Final Concentration (µM)
5x Phusion buffer	1x
dNTPs	200
Hot Start II High Fidelity DNA polymerase	1 unit
Forward primer (Table 3)	0.2
Reverse primer (Table 3)	0.2

For nested PCRs, 1 µl of first-round PCR product was used as the template for the subsequent PCR. DNA was separated according to size by agarose gel electrophoresis (standard methods) on a 1 % agarose gel containing 2.5 µM ethidium bromide, extracted from the gel and purified (QIAquick gel extraction kit, Qiagen). The sequence of PCR products was established by capillary sequencing (GATC Biotech) using eight primers (5-13, Table 3).

Site-directed mutagenesis was carried out using the QuikChange II XL site-directed mutagenesis kits (Agilent) according to manufacturer's directions (for primers see Table 3).

2.2.2 Detection of IFITM Gene Expression in Different Chicken Tissues

Tissues were removed from three-week-old specific pathogen-free (SPF) Rhode Island red chickens, specifically thymus, spleen, bursa of Fabricius, caecal tonsil, gastro-intestinal tract, trachea, bone marrow, brain, muscle, heart, liver, kidney, lung,

and skin. RNA was extracted by Karen Billington (Pirbright Institute) using an RNeasy minikit. Subsequent DNase treatment and reverse transcription was carried out at the Wellcome Trust Sanger Institute, using oligodT primers and SuperScript III reverse transcriptase (Life Technologies). The cDNA from each tissue was amplified by PCR using primer pairs 29-34 (Table 3).

2.2.3 Detection of IFITM1, 2, and 3 in Macrophages

RNA was extracted from human monocyte-derived macrophages (MDMs) and RT-PCR carried out using a 2-step protocol using SuperScript III Reverse Transcriptase (Life Technologies), Phusion High-Fidelity DNA Polymerase (Thermo Scientific), and primers 23-28 (Table 3). Plasmids containing the non-codon-optimised version of each IFITM gene were used as discriminatory controls for each reaction.

2.2.4 Quantification of IFITM1, 2, and 3 mRNA in Human Cell Lines

The endogenous levels of *IFITM1, 2*, and *3* mRNA in numerous human cell lines were detected by QuantiTect SYBR green qRT-PCR (Qiagen) using primers 23-28 (Table 3), and an Agilent MX3005P. The following thermocycling conditions were used: 30 min at 50 °C; 15 min at 95 °C; 35 cycles of 15 s 94 °C, 30 s 60 °C, 30 s 72 °C.

Five standards from 10^7 – 10^3 copies were made using plasmids encoding the non-optimised transcripts of human *IFITM1, 2, and 3*, using the following formula:

$$\left(\frac{X\text{g DNA}}{\text{plasmid length}\cdot 660}\right)\cdot 6.022\times 10^{23} = Y\text{molecules}$$

The total RNA was extracted from a known number of cells and the total amount of RNA extracted was recorded (RNeasy minikit). 100 ng of RNA was used as a template per qRT-PCR reaction. Using the standards for each transcript, the quantity of transcript was determined relative to the standard curve for the 100 ng input RNA. The number of copies per cell was estimated by dividing the total number of cells by the total RNA extracted, multiplied by 100. This gave the equivalent number of cells that produced 100 ng of RNA and from this the copies per cell was inferred.

IFITM3_004 was detected using primers 7 and 20 (Table 3) and the same thermocycling conditions.

2.2.5 Plasmid Preparation

Top10 competent cells (Life Technologies) were transformed with plasmid DNA according to the manufacturer's guidelines. For transformation of newly ligated DNA, 2 µl of ligation reaction and 50 µl of competent cells were used. Cells were diluted 10 fold and 100 µl spread onto selective antibiotic LB agar plates and incubated overnight at 37 °C.

For small-scale preparations of plasmid DNA, single ampicillin-resistant colonies were picked from each LB agar plate and used to inoculate 5 ml of LB media in a 50 ml falcon tube. Cultures were left overnight in a shaking incubator at 37 °C and 200 revolutions per minute (rpm). The culture was centrifuged (2 ml, 10000 *g*, 3 min) and the plasmid DNA extracted (QiaPrep spin mini-prep kit, Qiagen) following the manufacturer's protocol. The sizes of new plasmids were checked using restriction enzyme digests. Digests were carried out in 20 µl, and incubated at 37 °C for 2 h. All enzymes and appropriate buffers (New England Biosciences [NEB]) were used under reaction conditions advised by the manufacturer.

DNA fragments were cloned by ligation into pBNHA (Figure 20). All ligation reactions were carried out in a 10 µl reaction volume and contained 0.5 volumes of 2x T4 DNA Ligase Buffer and 0.05 volumes of T4 DNA Ligase (2000 units/µl) (NEB), incubated overnight at 4 °C. Sequencing was carried out using primers 14 and 15 (Table 3).

2.2.6 Constructing Lentiviral Plasmids Containing IFITM3 Coding Sequence

DNA sequences for chicken and human IFITMs were codon optimised for expression in human cells and synthesised by GeneArt. The transgenes were flanked by a BamHI and NotI restriction enzyme target sequence. The insert was digested from each GeneArt plasmid and ligated into a lentivirus expression vector, pBNHA (Figure 20) that encodes a C-terminal HA tag. Capillary sequencing (using SFFV_F and WPRE_R primers [Table 3]) was carried out to check the integrity of the sequences and ensure that the HA tag was in frame with the rest of the protein sequence.

2.3 Cell Culture

2.3.1 Maintenance

Adherent cell lines HEK293-T and MDCK were grown in Dulbecco's modified Eagle's

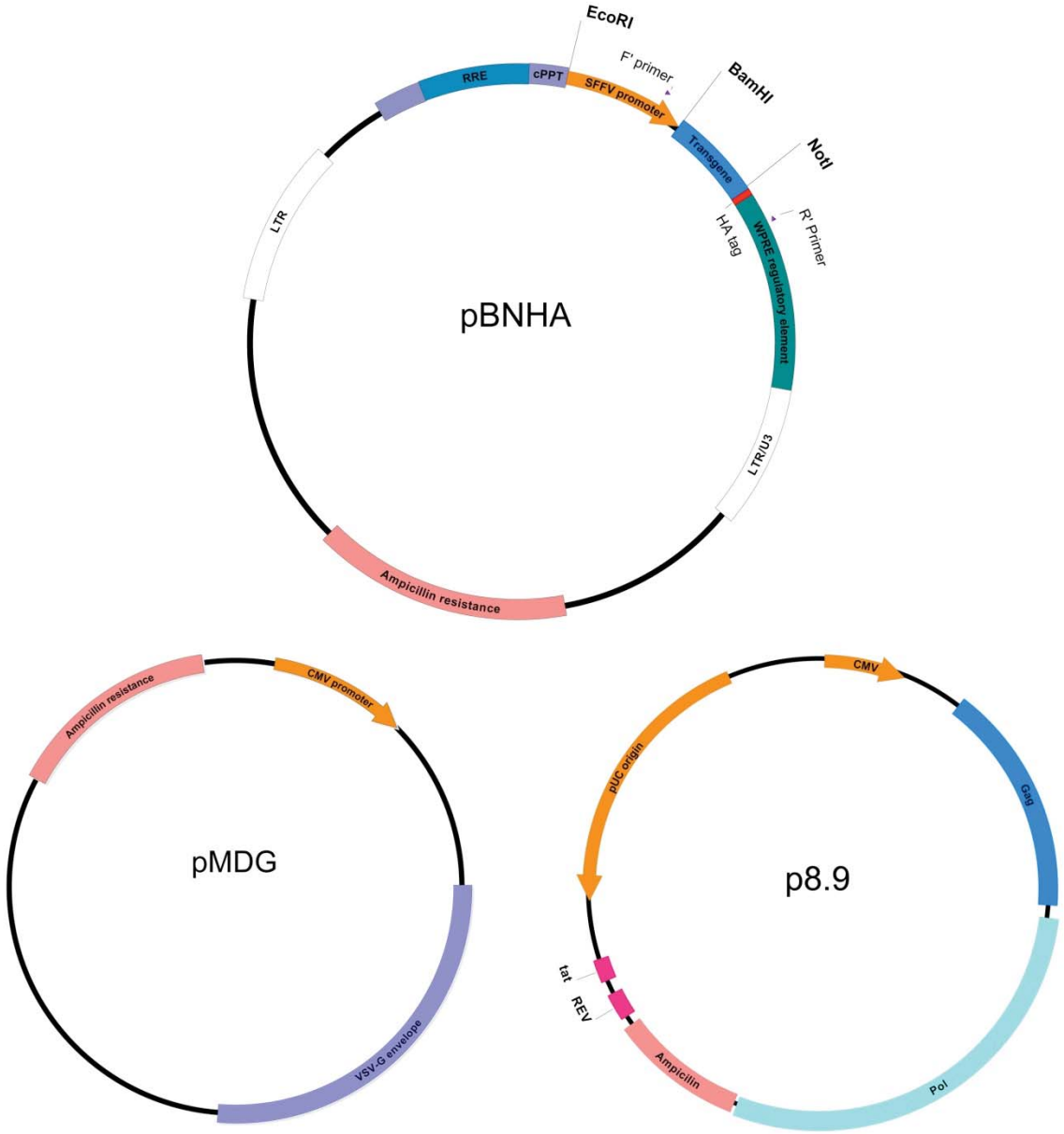

Figure 20: Expression vectors for generating lentiviruses

The 10 kb pBNHA lentiviral vector is based on pSIN-BNHA[235], and has restriction enzyme sites BamHI and NotI for inserting a transgene into the plasmid. The transgene is driven by the spleen focus-forming virus (SFFV) promoter and a C-terminal HA tag is added to the transgene. Forward and reverse primers used to sequence the transgene map to regions indicated in the promoter and Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element (WPRE). The packaging plasmid (p8.9) and envelope plasmid (pMDG) are also shown, which are required to produce lentivirus particles.

medium (DMEM, Life Technologies) and A549s were grown in F-12 media (Life Technologies). All media was supplemented with 10 % heat-inactivated foetal bovine serum (FBS, Biosera). Cells were passaged 1:6 or 1:10, twice a week depending on their density. Suspension lymphoblastoid cell lines (LCLs) were grown in RPMI 1640 (Life Technologies) with 10 % FBS. Primary airway epithelial (PAE) cells (ATCC-PCS-301-010) were grown in airway epithelial cell basal medium (ATCC-PCS-301-040) supplemented with small airway epithelial cell growth kit (ATCC-PCS-301-030, all LGC Standards). All cells were maintained at 37 °C in 5 % CO_2.

2.3.2 Freezing Cells

Cell were centrifuged at 200 *g* for 3 min and resuspended at $2x10^6$ cells/ml in chilled DMEM or F-12 media (20 % FBS). An aliquot of 500 µl was transferred to a cryotube (Greiner Bio-One) on ice. An additional 500 µl of chilled DMEM of F-12 (20 % FBS, 17.5 % DMSO) was layered on top before the cells were gradually cooled to -80 °C in an isopropanol-containing cryobox overnight before transfer to liquid nitrogen.

2.3.3 Thawing Cells

Cell were removed from liquid nitrogen and thawed rapidly at 37 °C. The aliquot was added to 8 ml of DMEM or F-12 media (10 % FBS) and centrifuged at 150 *g* for 3 min. The supernatant was removed and the cells resuspended in 8 ml of culture media and transferred to a culture vessel.

2.3.4 Single Cell Cloning

All wells of a clear 96 well plate were filled with 100 µl of culture media, except A1. 100 µl of cell suspension ($2x10^4$ cells/ml) was added to well A1 and B1, followed by a 1:2 serial dilution out to well H1. An additional 100 µl of culture media was added to all wells in column 1 and another serial dilution was carried out along each row of the plate to column 12. A further 100 µl of media was added to all wells in the 96 well plate to bring the final volume of each well to 200 µl before incubating the plate at 37 °C for 4-5 days. Wells with only 1 clone in them were marked and allowed to grow for a further 2 days. Cells were harvested into a 24 well plate and allowed to reach confluence. Cells were prepared for flow cytometric analysis (section 2.14) using an anti-HA antibody conjugated to FITC (1:400, A190-108F, Bethyl Laboratories) and those cells with a high level of HA tag expression were transferred to T25 flasks.

2.4 Confocal Microscopy

Cells were seeded at $1x10^5$/well on coverslips in a 12 well plate one day prior to transfection with an IFITM-encoding plasmid (1 µg DNA with 3 µl of fugene [Promega]). Cells were fixed with 100 % methanol for 10 min followed by blocking in 1 % BSA for 30 min. The HA epitope was targeted by an anti-HA tag antibody conjugated to Alexafluor 550 (1:500, abcam) and endosomes were visualised by a Lamp 1 antibody with human (ab25630, 1:1000 abcam) or chicken (LEP100 IgG, Developmental Studies Hybridoma Bank 1:400) specificity, followed by incubation with a secondary antibody conjugated to Alexafluor 488 (1:500 abcam).

2.4.1 Image Analysis

To calculate the Pearson's R-value and Mander's correlation coeficients (M1 and M2), individual cells were segregated and then analysed using the JACoP plugin on ImageJ software[236]. For M1 and M2 values, a Costes' automatic threshold was applied (as described[236]). For the calculation of the relative area of yellow, red and green signals, images were initially split into the red and green component channels. These two images were then processed with the AND function in ImageJ, producing an image of pixels where only both red AND green are present. This image was subject to a manual threshold to only observe cellular structures and remove any background noise. The pixel area was then calculated and these pixels defined as 'yellow'. These 'yellow' pixels were then super-imposed to the red and green single channel images, and removed from each of these. This work was carried out by Stuart Weston (University College London).

2.5 Immunohistochemistry

Lung biopsies were surgical specimens taken from the normal part of the lung whilst patients had a lobectomy for lung cancer (IRB reference number: UW 04-234 T/556). Lobes were fixed in paraffin and 1 mm cross-sections taken. Staining of IFITM3 was carried out by Kevin Fung (Department of Pathology, University of Hong Kong) using an anti-IFITM3 antibody (H00010410-M01, Abnova).

The sections were microwaved in 10 mM citrate buffer (pH 6) at 95 °C for 10 min. Sections were cooled to room temperature and washed with water. Blocking was

performed with 3 % H_2O_2 in tris buffered saline (TBS) for 15 min. Subsequently, sections were washed in running water, followed by TBS.

Further blocking of endogenous biotin or biotin receptors was carried out with Avidin / biotin blocking system (SP-2001, Vector Lab) for 15 min of each of the reagent A and reagent B (see manufacturer's instructions). Slides were washed three times with TBS, 5 min each. Sections were blocked again with 10 % Normal Goat Serum (NGS) (10 min, room temperature [RT]) followed by incubation with the primary antibody (1/1000 in 10 % NGS) for 1 h at RT. Slides were washed again with TBS, three times, 5 min each.

The tissue was then incubated a with biotinylated goat anti-mouse antibody (115-065-146, Jackson) at 1/500 for 30 min, followed by a further three TBS washes (as previously), before incubation with the ABC complex (ABC kit PK-6100, Vector) at 1/50 for 30 min. Sections were washed again with TBS before developing in 3,3'-diaminobenzidine (DAB) for 4 min. Slides were washed again in water and the nuclei stained with Mayer's Haematoxylin. Excess dye was washed off with water and left to air-dry before being mounted (Permount, Fisher Scientific).

2.6 Making and Titrating Lentivirus Stocks

Lentivirus stocks were made by a three plasmid transfection of HEK293-T cells, grown in a 10 cm dish. OptiMEM (200 µl, Gibco) was mixed with 10 µl of Fugene-6 (Roche). The DNA for transfection was made up in a final volume of 15 µl Tris-EDTA (TE), containing 1 µg of a gag-pol expressing vector (p8.91), 1 µg of a VSV-G expressing vector (pMDG) and 1.5 µg of vector expressing the transgene (pBNHA) (Figure 20). The DNA was added to the OptiMEM solution and incubated for 15 min. Once the media was removed from the cells and replaced with 8 ml of DMEM, 10 % FBS, the DNA mixture was added dropwise to the cells.

After 24 hours (h) at 37 °C and 5 % CO_2 the media was removed and replaced with 8 ml DMEM, 10 % FBS, and incubated for a further 24 h. Packaged virus was harvested at 48 and 72 h after transfection by collecting the supernatant and filtering using a 0.45 µM filter (Millex). Aliquots (1 ml) were frozen down at -80 °C.

Lentivirus stocks were concentrated using a sucrose cushion. For each viral sample, 500 µl of 20 % filter-sterilised sucrose (in phosphate buffered saline [PBS]) was overlaid with 800 µl of lentiviral supernatant in a 1.5 ml Eppendorf tube and centrifuged for 1 h at 20000 *g* at 4 °C. The liquid was aspirated, leaving behind an invisible pellet that was resuspended in 100 µl of RIPA buffer. Western blots (section 2.17) using an anti-p24 antibody (Abcam) were carried out on 20 µl of neat, 1:3 and 1:9 dilutions of the concentrated virus along with a sample of pBNHA_GFP (green fluorescent protein [GFP] expressing lentivirus). One well of $1x10^5$ HEK293-T cells was also transduced with an equivalent amount of the GFP virus and analysed by flow cytometry after 24 h to relate the intensity of the western blot band to the number of infected cells to give an estimate of biological viral titre.

2.7 siRNA Knock-Down Studies

DF-1 chicken cells were seeded in DMEM (10 % FBS) at $5x10^4$ cells/well in a 24 well plate and transfected with an siRNA against chIFITM3 (9) or a non-specific siRNA (UUCUCCGAACGUGUCACGUGU) using Lipofectamine RNAiMax (Life Technologies) (15 pmol siRNA:1.5 µl Lipofectamine/well) 48 h prior to IFN stimulation. The cells were stimulated by addition of either 200 ng/ml of chicken IFN-γ (Kingfisher biotech #RP0115c) or chicken IFN-α (AbD serotec #PAP004) for a further 24 h or infected with IAV (A/WSN/1933 [WSN/33]) for 1 h at an MOI of 0.1. RNA was extracted according to the manufacturer's instructions (RNeasy minikit, Qiagen). RT-PCR was performed (QuantiTect Multiplex RT-PCR kit, Qiagen) using probes and primers from ABI (chicken GADPH; 4448489 and chicken_IFITM3; custom assay). Influenza infection was measured according to 2.10 to determine cell infection.

2.8 Interferon Stimulation Experiments

LCLs were seeded at a density of $5x10^5$ cells per well of a 6 well plate, 24 h prior to the addition of $5x10^7$ units of IFN2αb per well (Source Bioscience). After a 24 h incubation the media was removed and the cells washed in PBS, before resuspension in 300 µl RIPA buffer (for protein analysis, see below), 350 µl RLT (for

RNA analysis, RNeasy mini kit) or 200 µl PBS (for DNA extraction, QIAmp DNA mini kit). To amplify the full-length IFITM3 transcript and the alternative transcript IFITM3_004, primers 23 and 24 or 7 and 20 (Table 3) were used, respectively, in downstream RT-PCR (2.2.4).

2.9 Cellomics Fluorescent Cell Analysis

Cells were seeded sparsely ($3x10^3$/well of a clear 96 well plate) and infected with a GFP expressing lentivirus. 48 h later cells were washed in 100 µl of PBS and fixed with 4 % v/v paraformaldehyde (USB) for 20 min. Cells were washed with 100 µl of PBS/Hoechst solution (Life Technologies, 200 ng/µl) and a plate seal adhered. The cells were analysed to determine the proportion of GFP expressing cells (Cellomics ArrayScan V^{TI} [Thermofisher], using the Target Activation bioapplication in CellomicsScan software).

2.10 Influenza Infection Assays

DF-1 or A549 cells were seeded at $2x10^5$/well of a 24 well plate 24 h prior to infection. IAV (WSN/33) was added at an MOI of 1 and cells were returned to the incubator for 1 h. Cells were harvested and treated according to the flow cytometric analysis protocol (2.14). An anti-NP antibody conjugated to FITC (1:1000, ab20921, Abcam) was used to determine cell infection.

2.11 Luciferase Reporter Infection Assays

Cells were seeded at $3x10^3$/well in a white 96 well plate and incubated for 24 h at 37 °C. An appropriate volume of pseusdotyped virus expressing the capsid of an influenza virus and a luciferase reporter gene was added to the cells and incubated for 48 h at 37 °C. The cells were removed from the incubator to reach room temperature before 50 µl of Bright-Glo™ reagent (Promega) was added to each well. The cells were allowed to lyse for 2 min before the level of luciferase activity was measured using a FLUOstar omega plate reader (BMG labtech).

2.12 Dual-Luciferase Signalling Reporter Assays

Cells were seeded at $2x10^4$/well in a white 96 well plate and incubated for 24 h at 37 °C. Cells in each well were transfected with 2 ng of transfection control plasmid (Renilla), 8 ng of Firefly luciferase reporter plasmid (NF-κB, IFNβ or ISRE) and 25 ng of pcDNA_MAVS/IFITM/TRIM5α/Tetherin. Control and reporter plasmids were a kind gift from Jeremy Luban, Adam Fletcher and Stuart Neil. A plasmid encoding a mutant tetherin protein (Y6.8A) was used as a reduced function control (gift of Stuart Neil) and an empty vector was used as a negative control. Transfection was carried out using lipofectamine 3000 (Life technologies) and OptiMEM, according to the manufacturer's instructions. Cells were incubated for 24 h and either TLR agonists applied (poly I:C or CpGs, both Invivogen) for 6 h or viruses were applied (influenza A/WSN/1933, MOI 1, or MLV-A (ATCC-VR1450) for 24 h. The cells were removed from the incubator to reach room temperature before 50 µl of Dual-Glo™ reagent (Promega) was added to each well. The cells were allowed to lyse for 10 min before the level of Firefly luciferase activity was measured using a FLUOstar omega plate reader (BMG labtech). Subsequently the reaction was stop by adding the Stop-Glo™ reagent, which quenched the reaction and provided the substrate for Renilla luciferase. The cells lysates were incubated for a further 10 min and then the Renilla luciferase activity was measured.

2.13 Quantifying Influenza Virus Using a Plaque Assay

MDCK cells were seeded ($6x10^6$ cells/well in a 6 well plate) and incubated at 37 °C until confluent. Six serial dilutions were carried out using 55.5 µl of viral supernatant in 500 µl of serum free (SF) DMEM (10^{-1} to 10^{-6}). The media was removed from the plate and the cells washed with SF DMEM. An aliquot of each viral dilution (250 µl) was added to cells and incubated for 1 h at 37 °C. Subsequently 1 ml of overlay media (Table 5) was added to each well and incubated for 2-3 days at 37 °C. The media was removed and 1 ml of formal saline (10 % v/v formaldehyde [Sigma] in PBS) was added and the cells left at room temperature for 20 min. Toluidine blue stain (Sigma) was added to the wells and left for 30 min until clear plaques in the monolayer could be seen. The number of plaques per well were counted and the average number of plaque s over the dilutions determined. This number was

Table 5: Overlay media for plaque assays

Chemical	Volume for 6-well plate
DMEM (Life Technologies)	6.69 ml
L-glutamine (Life Technologies)	125 µl
2.5 % avicel (FMC Biopolymer)	6.69 ml
TPCK trypsin (Worthington Biochemical)	13.25 µl
7.5 % Bovine serum albumin (BSA, Life Technologies)	187.5 µl

multiplied by 4 to calculate the number of plaque-forming units per ml (pfu/ml). This number was multiplied by to the power of the dilution factor to get the titre of the final virus titre in pfu/ml.

2.14 Flow Cytometric Analysis

Cells were removed from the plate using 300 µl of 0.25 % Trypsin-EDTA (Life Technologies), neutralised with 300 µl of cell culture media (10 % FBS), and pooled with the floating cells in the supernatant removed from the wells. The cells were spun at 2000 *g* for 5 min, the pellet resuspended in 100 µl of PBS and transferred to 96 well v-bottomed plate. The plate was centrifuged again, and the cells fixed and permeablised in 100 µl of Cytofix/Cytoperm™ buffer (Becton Dickinson) and washed according to manufacturer's guidelines. The cells were resuspended in the primary antibody and incubated for 1 h at 4 °C, followed by two rounds of washing. Cells were subsequently resuspended in the secondary antibody conjugated to a fluorescent protein and incubated in the dark for 1 h, unless the primary antibody had a conjugated fluorescent marker. Cells were washed again, resuspended in 300 µl of PBS before analysis by flow cytometry (FACSCalibur II, Becton Dickinson). Data was analysed using BD CellQuest Pro software.

If cells were expressing GFP, they were fixed with 4 % v/v paraformaldehyde (USB) for 20 min, washed twice with PBS, and resuspended in 300 µl of PBS prior to analysis by flow cytometry as above.

2.15 Nucleotide Extraction from Fixed Tissue Samples

DNA and RNA were extracted from formalin fixed paraffin embedded human lung tissue samples (kind gift of Prof. John Nicholls, University of Hong Kong) using the QIAamp DNA FFPE Tissue Kit and the RNeasy FFPE Kit respectively (both Qiagen). Paraffin wax was removed by immersion in xylene according to the manufacturer's instructions. Ethical approval was given by the Institutional review board of the University of Hong Kong (UW 04-234 T/556).

2.16 Phylogenetic Analysis

Sequences were aligned at the amino acid level using ClustalW. Bayesian consensus trees were inferred using mrBayes version 3.2.1, under a GTR+Γ_4 substitution model. Two sets of three MCMC chains, each one million states, was used to sample the posterior tree space, with consensus trees generated following a 25 % burn-in. Trees were formatted using FigTree v 1.4.0.

2.17 Protein Manipulation

Western blotting was used to determine the relative levels of proteins expressed by cells in culture. Cells were lysed and proteins solubilised by resuspension in RIPA buffer (Sigma) containing 1x Halt protease inhibitors (Pierce). Protein concentration was determined using the bicinchonic acid assay (BCA) protein kit (Pierce). Unless otherwise stated, loading dye was added to 10 µg of protein and incubated at 95 °C for 5 min. The sample was loaded onto a 4-20 % Mini-PROTEAN TGX gel (BioRad) along with 5 µl of Precision Plus Protein Kaleidoscope standards (BioRad). The proteins were separated according to size using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) at 250 V for 25 min. Proteins were transferred to a nitrocellulose membrane (Trans-Blot Turbo Midi Nitrocellulose) using the Trans-Blot Turbo System according to the manufacturer's guidelines.

Membranes were blocked with blocking buffer (5 % w/v milk powder [Marvel]) in PBS-T (0.05 % tween-20 in PBS) on a rocker for 1 h. The appropriate primary antibody was diluted in 10 ml of blocking buffer and used to blot the membrane for

1 h. For detection of HA-tagged IFITM proteins an anti-HA antibody was used (1:1000, ab18181, Abcam).

This was followed by washing in PBS-T for 30 min before incubation with the secondary antibody (either goat anti-mouse [1:4000, p0447, Dako] or swine anti-rabbit [1:5000, p0399, Dako]) in 10 ml of blocking buffer for 1 h. The membrane was washed again and 5 ml of ECL Plus chemiluminescent substrate added to each membrane according to manufacturer's guidelines (Amersham). The membrane was then exposed to a sheet of Hyperfilm (Amersham).

2.18 Co-Immunoprecipitation

Co-Immunoprecipitation (co-IP) assays were used to identify interacting partners of IFITM proteins. After harvesting in gentle lysis buffer, the protein supernatant was mixed with either magnetic Dynabeads (small-scale) or agarose beads (large-scale).

2.18.1 Magnetic Dynabeads

The co-IP was carried out using the magnetic Dynabeads® Protein A Immunoprecipitation Kit (Life technologies) onto which an anti-HA antibody (ab18181) was conjugated according to the manufacturer's instructions. Peptides were eluted using the manufacturer's elution buffer (glycine) or 100 µl of free HA peptide (Sigma, 5 mg/ml).

Cross-linking of the antibody to the magnetic bead was carried out using both Bis(sulfosuccinimidyl)suberate (BS^3, Thermofisher) and dimethyl pimelimidate (DMP, Sigma). The Dynabeads were resuspended in 250 µl of 5 mM of BS^3 in conjugation buffer (20 mM NaK, 0.15 M NaCl) and incubated at room temperature for 30 min with rotation. The cross-linking was quenched by addition of 12.5 µl of 1 M Tris HCl and incubation for 15 min at room temperature. The beads were washed in 200 µl PBS-T and the co-IP proceeded according to the manufacturer's instructions. Alternatively, the beads were washed in 500 µl of 0.1 M sodium citrate (pH 5) followed by incubation with 2 µg of anti-HA antibody (ab18181) in 100 µl 0.1 M sodium citrate for 40 min with rotation. The beads were washed in 0.1 M sodium citrate-0.01 % Tween-20 and the supernatant removed. Subsequently the beads were resuspended in 1 ml of 20 mM DMP (in 0.2 M triethanolamine) for 30 min with rotation to cross-link the

antibody and beads. The supernatant was discarded and the reaction quenched by addition of 1 ml 50 mM Tris-HCl for 15 min with rotation.

2.18.2 Pre-Bound Anti-HA Beads

For large-scale preps, pre-bound anti-HA agarose beads (ab1233, Abcam) were used to precipitate IFITM3_HA. 1 ml of the lysate was loaded onto 150 µl of beads and allowed to rotate for 5 h at 4 °C. The beads were centrifuged at 3000 *g* for 3 min at 4 °C and the supernatant removed and kept for analysis for Western blot. The beads were washed twice in gentle lysis buffer and centrifuged as before (washes also kept for analysis). The bound proteins were eluted with 100 µl of free HA peptide (Sigma, 5 mg/ml).

2.19 Ethics and Sampling of Patients with A/H1N1/09

Patients with confirmed seasonal IAV, influenza B virus or pandemic IAV (A/H1N1/09) infection who required hospitalisation in England and Scotland between November 2009 and February 2011 were recruited into the MOSAIC and GenISIS studies. Patients with significant risk factors for severe disease, and patients whose daily activity was limited by co-morbid illness, were excluded. 53 patients, 29 male and 24 female, average age 37 (range 2–62) were selected. 47 (89 %) had no concurrent co-morbidities. The remaining six had the following comorbid conditions: hypertension (three patients), alcohol dependency and cerebrovascular disease (one patient), bipolar disorder (one patient) and kyphoscoliosis (one patient). Four patients were pregnant.

Consent was obtained directly from competent patients, and from relatives/friends/welfare attorneys of incapacitated patients. The GenISIS study was approved by the Scotland ‘A’ Research Ethics Committee (09/MRE00/77) and the MOSAIC study was approved by the NHS National Research Ethics Service, Outer West London REC (09/H0709/52, 09/MRE00/67). Anonymised 9 ml EDTA blood samples were transported at ambient temperature. DNA was extracted using a Nucleon Kit (GenProbe) with the BACC3 protocol. DNA samples were re-suspended in 1 ml TE buffer pH 7.5 (10 mM Tris-Cl pH 7.5, 1 mM EDTA pH 8.0). This work was carried out by members of the MOSAIC and GenISIS consortia.