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Bat and pig Interferon-Induced Transmembrane Protein 3 restrict cell entry by influenza virus and lyssaviruses
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Abstract:	Interferon-induced transmembrane protein 3 (IFITM3) is a restriction factor which blocks cytosolic entry of numerous viruses that utilise acidic endosomal entry pathways. In humans and mice, IFITM3 limits influenza-induced morbidity and mortality. Although many IFITM3-sensitive viruses are zoonotic, whether IFITMs function as antiviral restriction factors in mammalian species other than humans and mice is unknown. Here, IFITM3 orthologues in the microbat Myotis myotis and the pig (Sus scrofa domesticus) were identified using rapid amplification of cDNA ends. Amino acid residues known to be important for IFITM3 function were conserved in the pig and bat orthologues. Ectopically-expressed pig and microbat IFITM3 co-localised with transferrin (early endosomes) and CD63 (late endosomes/multivesicular bodies) and trafficked from the plasma membrane into endosomes following live cell staining. Pig and microbat IFITM3 restricted cell entry mediated by multiple influenza HA subtypes and lyssavirus G proteins. Expression of pig or microbat IFITM3 in A549 cells reduced influenza virus yields and nucleoprotein expression. Conversely siRNA knockdown of IFITM3 in pig NPTr cells and primary microbat cells enhanced virus replication, demonstrating that these genes are functional in their species of origin at endogenous levels. In sum, we show that IFITMs function as potent broad-spectrum antiviral effectors in two mammals - pigs and bats - identified as major reservoirs for emerging viruses.

1   **Bat and pig Interferon-Induced Transmembrane Protein 3 restrict cell entry by**

2   **influenza virus and lyssaviruses**

3

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27

28   **Summary**

29

30   Interferon-induced transmembrane protein 3 (IFITM3) is a restriction factor which blocks

31   cytosolic entry of numerous viruses that utilise acidic endosomal entry pathways. In humans

32   and mice, IFITM3 limits influenza-induced morbidity and mortality. Although many IFITM3-

33   sensitive viruses are zoonotic, whether IFITMs function as antiviral restriction factors in

34   mammalian species other than humans and mice is unknown. Here, IFITM3 orthologues in

35   the microbat *Myotis myotis* and the pig (*Sus scrofa domesticus*) were identified using rapid

36   amplification of cDNA ends. Amino acid residues known to be important for IFITM3 function

37   were conserved in the pig and bat orthologues. Ectopically-expressed pig and microbat

38   IFITM3 co-localised with transferrin (early endosomes) and CD63 (late

39   endosomes/multivesicular bodies). Pig and microbat IFITM3 restricted cell entry mediated by

40   multiple influenza HA subtypes and lyssavirus G proteins. Expression of pig or microbat

41   IFITM3 in A549 cells reduced influenza virus yields and nucleoprotein expression.

42   Conversely siRNA knockdown of IFITM3 in pig NPTr cells and primary microbat cells

43   enhanced virus replication, demonstrating that these genes are functional in their species of

44   origin at endogenous levels. In sum, we show that IFITMs function as potent broad-spectrum

45   antiviral effectors in two mammals – pigs and bats – identified as major reservoirs for

46   emerging viruses.

47

48

Introduction

50

51 Restriction factors are germline-encoded proteins that function in a cell-autonomous manner

52 to suppress virus replication. The interferon-induced transmembrane (IFITM) proteins are a

53 family of small interferon (IFN)-stimulated proteins, which affect diverse cellular processes

54 (reviewed in (Siegrist *et al.*, 2011)) and were recently identified as antiviral restriction factors

55 which inhibit cell entry of multiple pathogenic viruses (Brass *et al.*, 2009).

56

57 To date, IFITMs have been reported to restrict the enveloped viruses influenza A, West Nile

58 Virus and Dengue Virus (*Flaviviridae*), SARS-coronavirus, Ebola and Marburg viruses

59 (*Filoviridae*), Vesicular Stomatitis Virus (VSV) and lyssaviruses (*Rhabdoviridae*), HIV-1 and

60 several species of *Bunyaviridae,* as well as a non-enveloped orthoreovirus (*Reoviridae*)

61 (Anafu *et al.*, 2013; Brass *et al.*, 2009; Huang *et al.*, 2011; Jia *et al.*, 2012; Lu *et al.*, 2011;

62 Mudhasani *et al.*, 2013; Smith *et al.*, 2013; Weidner *et al.*, 2010). The common feature of

63 IFITM-sensitive viruses appears to be their dependence on acidic endosomal entry

64 pathways, either for proteolytic cleavage, or pH- or protease-dependent activation of viral

65 entry proteins into their fusogenic form. Accordingly, pseudotyped retroviruses expressing

66 heterologous surface envelope proteins recapitulate the IFITM sensitivity of the authentic

67 virus from which the envelope protein derives, and have been widely used to study IFITM

68 biology (Brass *et al.*, 2009; Feeley *et al.*, 2011; Huang *et al.*, 2011). The IFITMs localise to

69 membranes of late endosomes and lysosomes and prevent the release of viral particles from

70 these compartments into the cytosol (Feeley *et al.*, 2011).

71

72 The human *IFITM* gene family comprises *IFITM1, -2, -3, -5,* all of which possess antiviral

73 activity and cluster together on chromosome 11, as well as *IFITM10* whose function remains

74 unknown. Mice possess orthologues of all the human *IFITM* genes and two additional

75 genes, *Ifitm6* and *Ifitm7*. IFITM1-3 are expressed in a wide range of tissues (Bailey *et al.*,

76 2012; Everitt *et al.*, 2013; Siegrist *et al.*, 2011) whereas IFITM5 expression is limited to

77 osteoblasts (Moffatt *et al.*, 2008). Of all the IFITMs, IFITM3 is the most potent anti-influenza

78 effector *in vitro* (Huang *et al.*, 2011). Ifitm3 has a critical role in limiting influenza-induced

79 morbidity and mortality in mice (Everitt *et al.*, 2012). Since the phenotype of influenza-

80 infected *Ifitm3*^{-/-} mice was indistinguishable from that of mice deleted for the entire locus

81 (comprising *Ifitm1,-2,-3,-5* and *-6*), Ifitm3 apparently dominates influenza resistance *in vivo*

82 *(Bailey et al., 2012)*. Moreover, the importance of IFITM3 was highlighted by reports showing

83 that an *IFITM3* allele (rs12252-C) is associated with enhanced disease severity caused by

84 pandemic influenza H1N1/09 (Everitt *et al.*, 2012; Zhang *et al.*, 2013b) and highly

85 pathogenic influenza H7N9 (Wang *et al.*, 2014) and is more frequent in Chinese than

86 Caucasian populations.

87

88 IFITM proteins comprise a relatively long hydrophilic N-terminal region, two hydrophobic

89 intramembrane domains (IM1 and IM2), separated by a conserved intracellular loop (CIL),

90 and a comparatively short hydrophilic C-terminal region. The IM1 and CIL together constitute

91 the CD225 domain, a functionally poorly-defined domain shared by >300 members of the

92 CD225/pfam04505 protein superfamily. Several alternative topologies have been proposed

93 for the IFITMs (Chen *et al.*, 1984; Li *et al.*, 2013; Takahashi *et al.*, 1990; Yount *et al.*, 2012),

94 but the most recent study suggests that murine Ifitm3 is a type II transmembrane protein,

95 comprising an intracellular N-terminus, an extracellular C-terminus and a membrane-

96 spanning 'IM2' domain (Bailey *et al.*, 2013). IFITM function is regulated by several post-

97 translational modifications. S-palmitoylation of IFITM3 on three membrane proximal cysteine

98 residues enhances membrane affinity and antiviral activity against influenza (Yount *et al.*,

99 2012; Yount *et al.*, 2010). Conversely, lysine-linked ubiquitination decreases IFITM3's co-

100 localisation with endolysosomes and its antiviral potency (Yount *et al.*, 2012). The Y20

101 residue, which can be phosphorylated by the tyrosine kinase Fyn, is critical for targeting

102 IFITM3 to endolysosomes and restriction of endosome-dependent viruses (Jia *et al.*, 2012;

103 John *et al.*, 2013).

104

105 Current evidence indicates that IFITMs block viral entry to the cytosol by preventing fusion

106 between viral and host cell membranes. IFITMs multimerise via their IM1 regions (John *et*

107 *al.*, 2013) and increase membrane rigidity (Li *et al.*, 2013). This suggests a model in which

108 IFITMs either physically resist the deformation of the membrane by the viral fusion

109 machinery or hinder the lateral mobility of viral or cellular proteins within the membrane and

110 thereby block successful pore formation (John *et al.*, 2013; Li *et al.*, 2013; Perreira *et al.*,

111 2013). Amini-Bavil-Olyaee *et al.* recently reported that IFITM3 interacts with a protein

112 involved with cholesterol homeostasis, vesicle-membrane-protein-associated protein A

113 (VAPA), and thereby causes cholesterol accumulation in multivesicular bodies and late

114 endosomes which inhibits the fusion between virion and endosomal membranes (Amini-

115 Bavil-Olyaee *et al.*, 2013). However, a unifying mechanism to explain antiviral restriction by

116 IFITM proteins remains elusive (Perreira *et al.*, 2013; Smith *et al.*, 2014). Indeed recent

117 reports showing that IFITM3 can be co-opted to promote cell entry of human coronavirus

118 OC43 (Zhao *et al.*, 2014) and human papillomavirus (Warren *et al.*, 2014) suggest virus-

119 specific IFITM interactions.

120

121 Although the *IFITM* gene family is evolutionary conserved in vertebrates (Hickford *et al.*,

122 2012; Zhang *et al.*, 2012), it is unclear whether antiviral activity is also conserved among

123 vertebrate IFITMs. Here, we focused on the bat and pig since these hosts are particularly

124 relevant to the ecology of several IFITM-sensitive zoonotic viruses.

125

126

127

128 **Results**

129

130 **Sequence analysis of IFITM3 orthologues cloned from microbat and pig cells**

131

132 In vertebrates, the *IFITM-1, -2, -3* and -*5* genes cluster together in an *IFITM* locus flanked by

133 the *B4GALNT4* and *ATHL1* genes. It was not possible to assign pig and bat IFITM3

134 orthologues based on conserved synteny due the lack of a *B4GALNT4* orthologue in pigs,

135 gaps in the genome assemblies, and the low sequencing coverage of the bat genomes (2.6-

136 X for *Pteropus vampyrus* and 1.7-X for *Myotis lucifugus* at the time of analysis). Therefore,

137 to identify *IFITM* genes, rapid amplification of cDNA ends (RACE) was performed on a

138 newborn pig trachea cell line (NPTr) and primary lung fibroblasts from the greater mouse-

139 eared bat, *Myotis myotis*, a European species of microbat (*Vespertilionidae*). *M. myotis* was

140 selected since it is a known reservoir host for several highly pathogenic viruses (Amengual

141 *et al.*, 2007; Drexler *et al.*, 2011; Drexler *et al.*, 2012) and genomes are available for other

142 species from the same genus (Seim *et al.*, 2013; Zhang *et al.*, 2013a).

143

144 RACE using primers designed to the conserved central regions of compiled *IFITM*-like

145 sequences yielded several *IFITM* gene variants, of which the designated IFITM3-like

146 sequences were the most abundant. For the pig, the IFITM3-like sequence was the only

147 IFITM variant identified which had an N-terminal extension typical of IFITM2/3 proteins

148 (compare human IFITM1-3 in Fig. 1b). For the microbat, the sequence we assigned as

149 IFITM3 was the most frequent of several long IFITM variants (68% of sequenced clones)

150 and the only one encoding a double phenylalanine motif (F8/F9) conserved in the human

151 and pig IFITM3 orthologues but absent from human IFITM2 (Fig. 1b).

152

153 Full-length *IFITM3* cDNA sequences were obtained by RT-PCR, and introns were identified

154 by PCR using genomic DNA, thereby confirming that these are not intron-less expressed

155   pseudogenes. The transcript structure for pig and microbat IFITM3 was the same as for

156   other experimentally verified IFITM3 orthologues, comprising two exons, a single intron of

157   similar size to other *IFITM3* genes and a conserved exon-exon junction site (Fig. 1a). BLAST

158   searches revealed that the *IFITM3* sequence from NPTr cells is identical to the *Sus scrofa*

159   *IFITM3* reference sequence (NM_001201382.1), and the closest match for the cloned

160   microbat IFITM3 was a 'predicted *IFITM3*-like' gene from *M. lucifugus* (XP_006108229.1,

161   95% amino acid identity).

162

163   Multiple sequence alignments showed that amino acid residues which are functionally

164   important in murine and human IFITM3 are conserved in the cloned pig and microbat

165   orthologues (Fig. 1b). These include (i) three cysteine residues which are S-palmitoylated

166   (Yount *et al.*, 2010), (ii) several lysine residues modified by ubiquitination (Yount *et al.*, 2012)

167   (including K88 which can be monomethylated (Shan *et al.*, 2013)), (iii) the Y20 residue

168   critical for endosomal targeting (Jia *et al.*, 2012; John *et al.*, 2013), (iv) two phenylalanine

169   residues (F75 and F78) which mediate oligomerisation (John *et al.*, 2013) and (v) R85, R87

170   and Y99 shown to influence antiviral restriction (John *et al.*, 2013). The microbat and pig

171   IFITM3 proteins are most divergent from human IFITM3 at their N and C termini, and most

172   conserved in the central CD225 domain, a pattern shared with other orthologues (chicken

173   and mouse Ifitm3) and paralogues (human IFITM2 and IFITM1) (Fig. 1b).

174

175   **Microbat and pig IFITM3 localize to transferrin and CD63 positive endosomes**

176

177   To analyse the IFITM3 proteins, A549 cells (which express low levels of endogenous IFITM3

178   (Brass *et al.*, 2009)) were stably transduced to express C-terminally HA-tagged IFITM3 from

179   pig, microbat or human. Prior work has shown that C-terminal epitope tags do not affect

180   function or expression levels of IFITM3, and indicate that tagged constructs adopt the same

181   topology as the wild type protein (Bailey *et al.*, 2013).

182

183   Similar to human IFITM3, both pig and microbat IFITM3 had a punctate intracellular

184   distribution following cell fixation and permeabilisation (Fig. 2a-c) and co-localised with

185   endocytosed transferrin (early endosomes) and with CD63, a marker for late endosomes/

186   multivesicular bodies (MVBs), but not with the lysosomal marker LAMP1 (Fig 2a-c). There

187   was an enlargement of CD63 positive structures in cells expressing microbat or human

188   IFITM3 in comparison to the smaller CD63 positive vesicles seen in pig IFITM3-expressing

189   or untransduced A549 cells (Fig. 2b). Enlargement of CD63-containing compartments was

190   most marked in cells containing larger foci of IFITM3-HA staining. Microbat IFITM3-HA

191   sometimes co-localised with CD63 in 'hollow' ring-like structures (arrows in Fig. 2b).

192

193   **Endocytic uptake of microbat and pig IFITM3 from the plasma membrane**

194

195   To investigate the trafficking of pig and microbat IFITM3s, live cells were incubated with

196   FITC-conjugated anti-HA antibody and Alexa 546-conjugated transferrin prior to fixation.

197   When labeling was performed on ice, endocytosis was prevented as indicated by the weak

198   stippled transferrin signal, likely corresponding to clathrin-coated pits pre-internalisation (Fig.

199   3a). Under these conditions, IFITM3 from the three species was detected on the plasma

200   membrane, clearly highlighting filopodia and membrane ruffles, while no vesicular staining

201   was observed. In contrast, when labeling was performed at 37°C to allow endocytosis, anti-

202   HA staining showed that pig, microbat and human IFITM3 formed discrete puncta, which, as

203   previously observed, overlapped or were closely associated with transferrin-positive

204   endosomes (Fig. 3b). For all IFITM3s, internalization of the anti-HA antibody by live cells

205   identified larger, brighter puncta that were more widely distributed within the cytosol

206   compared to the HA staining seen previously following fixation and permeabilisation

207   (indicating that permeabilisation may cause some extraction of the membrane associated

208   IFITM3). In conclusion, pig and microbat IFITM3 share the following with their human

209   counterpart: (i) plasma membrane trafficking (ii) extracellular exposure of their C-termini

210 (allowing detection of the C-terminal HA tag in intact cells) and (iii) endocytic uptake from the

211 plasma membrane into the endosomal pathway.

212

213 **Microbat and pig IFITM3 restrict cell entry mediated by influenza HA**

214

215 To determine whether pig and microbat IFITM3 restrict influenza A virus entry, lentiviruses

216 expressing the HA proteins from diverse influenza subtypes were used to infect A549 cells

217 that were untransduced or had been transduced to express either human IFITM3, pig

218 IFITM3 (Fig. 4a) or microbat IFITM3 (Fig. 4b). Three independently cloned cell lines of the

219 pig and microbat IFITM3s markedly inhibited the infectivity of all influenza HA subtypes

220 tested, including both Group I and Group 2 HAs and highly pathogenic avian H5 and H7

221 (Fig. 4). Pig or microbat IFITM3 did not restrict viruses pseudotyped with the entry proteins

222 of the gammaretroviruses amphotrophic murine leukemia virus or Gibbon ape leukemia virus

223 (Fig. S1), consistent with previous studies on human IFITM3 (Brass *et al.*, 2009; Huang *et*

224 *al.*, 2011).

225

226

227 **Microbat and pig IFITM3 inhibit replication-competent influenza virus**

228

229 To assess the effect of the IFITM3 orthologues on replication-competent influenza virus,

230 untransduced A549 cells or cells expressing either human, pig or microbat IFITM3 were

231 infected with influenza A/WSN/33 (H1N1) and nucleoprotein (NP) expression was

232 quantitated using flow cytometry. Expression of either pig or microbat IFITM3 led to a

233 marked reduction in the proportion of NP positive cells for all cell clones (Fig. 5a). There was

234 slight variation in the degree of restriction between the three cell lines expressing pig IFITM3

235 (Fig. 5a and Fig. S2) despite comparable expression of IFITM3-HA seen by Western blot

236 (Fig. S2).

237

238 Stable expression of either pig, microbat or human IFITM3 in A549 cells significantly

239 reduced single cycle growth of influenza A/WSN/33 (Fig. 5c), consistent with reduced NP

240 expression measured in parallel (Fig 5b). Inhibition of virus yields was more profound

241 following low m.o.i. infection (>1 $\log_{10}$ for pig IFITM3 and >2 $\log_{10}$ for both microbat and

242 human IFITM3) relative to control GFP-expressing cells (Fig. 5d). A similar pattern of

243 restriction was also observed when NP expression was analysed after infections with an

244 avian-like swine influenza strain, A/swine/453/06 (H1N1) (Fig. 5b). Levels of the stably

245 expressed proteins were examined by Western blotting against the C–terminal HA epitope

246 tag, and showed that pig IFITM3, human IFITM3 and GFP-HA expression were comparable

247 while microbat IFITM3 was more highly expressed (Fig. 5c and Fig S3).

248

249 **Microbat and pig IFITM3 block cell entry mediated by lyssavirus entry proteins**

250

251 In light of data which suggest there may be virus-specific antiviral determinants of IFITM

252 (John *et al.*, 2013; Zhao *et al.*, 2014), it was of interest to explore the antiviral spectrum of

253 pig and microbat IFITM3, especially for viruses that naturally infect these species.

254

255 A549 cells stably expressing either pig, microbat or human IFITM3 were infected with

256 pseudotyped lentiviruses that expressed the envelope glycoproteins from isolates

257 representing different lyssavirus phylogroups, namely the rabies virus strain Evelyn Rokitniki

258 Abelseth (phylogroup 1), Mokola virus (phylogroup 2), Lagos bat virus (phylogroup 2) and

259 West Caucasian bat virus (phylogroup 3). Expression of IFITM3 from either microbat, pig or

260 human reduced infectivity mediated by all four lyssavirus entry proteins, in all cases by

261 approximately 2 $\log_{10}$ relative to control cells (Fig. 6). Thus, the pig and microbat IFITM3

262 orthologues inhibit cell entry mediated by multiple lyssavirus G proteins.

263

264 **IFITM3 activity in pig and microbat cells**

265

266   Finally, we addressed the contribution of IFITM3 to antiviral responses in pig and microbat

267   cells. Endogenous IFITM3 expression was measured using Taqman qRT-PCR designed to

268   specifically detect IFITM3 mRNA and not other IFITM paralogues identified in these cells by

269   RACE. Baseline levels of IFITM3 mRNA were readily detected in both the porcine NPTr cells

270   (Ct value IFITM3: 22.7; Ct value GAPDH 21.33) and in microbat lung fibroblasts (Ct value

271   IFITM3: 25.8; Ct value GAPDH 22.8). IFITM3 induction was assessed in response to the

272   dsRNA analogue polyI:C, a molecular pattern associated with viral infection which is

273   recognised by toll-like receptor 3 and induces Type I IFN in porcine (Provost *et al.*, 2012)

274   and bat cells (Biesold *et al.*, 2011; Omatsu *et al.*, 2008; Zhou *et al.*, 2011). PolyI:C addition

275   led to a 3.5-fold increase in pig IFITM3 and a 2-fold increase in microbat IFITM3 mRNA

276   levels (Fig. 7). The microbat cells were also stimulated via polyI:C transfection since in

277   pteropid bat lung cells (but not primary cells from other tissues) this enhanced IFNβ

278   induction relative to extracellular delivery of polyI:C (Zhou *et al.*, 2011). However,

279   transfection of polyI:C into microbat cells resulted in a similar degree of IFITM3 induction

280   (2.3-fold) (Fig. 7).

281

282   Next, the function of endogenous IFITM3 was assessed using siRNA designed using the

283   RACE sequence data to target IFITM3 and not other putative IFITM paralogues. siRNA

284   targeting IFITM3 or control non-targeting siRNAs were transfected into cells with polyI:C

285   induction of IFITM3. IFITM3 mRNA was quantified by qRT-PCR and the biological effect of

286   the knockdown assessed by infection with influenza A/WSN/33 (Fig. 8). Transfection of

287   siRNA against pig IFITM3 led to a 63% knockdown in IFITM3 mRNA (1.4 $\log_2$ fold change)

288   (Fig. 8a), a 3.4-fold increase in virus yields (Fig. 8b) and a 2-fold increase in the proportion

289   of influenza NP positive cells (Fig. 8c) relative to control siRNA transfected cells. In the

290   absence of polyI:C stimulation, IFITM3 knockdown in NPTr cells increased influenza NP

291   positive cells more (3.3-fold) (Fig. 8d) suggesting additional polyI:C induced genes

292   complement IFITM3 mediated influenza virus restriction. Microbat IFITM3 knockdown in the

293   absence of polyI:C stimulation (Fig. 8e-g) reduced IFITM3 mRNA levels by 60% (1.3 $\log_2$ fold

294 change) (Fig. 8e) and led to a 2-fold increase in infectious yields (Fig. 8f) and a 3-fold

295 increase in NP expression (Fig. 8g), relative to control siRNA transfection. Thus,

296 endogenous IFITM3 in pig tracheal NPTr cells and microbat lung cells restricts influenza

297 virus. Lastly, following siRNA knockdown of baseline IFITM3 in a microbat cell line,

298 overexpression of microbat IFITM3 significantly inhibited influenza NP expression (Fig. S4).

299

300 **Discussion**

301 Species differences in restriction factors can determine differential viral susceptibility

302 (Duggal & Emerman, 2012; Fadel & Poeschla, 2011; Kirmaier *et al.*, 2010; McNatt *et al.*,

303 2009). However, antiviral immunity in reservoir and spill-over hosts remains poorly

304 understood, although important for understanding viral emergence (Bean *et al.*, 2013). Here

305 we show that IFITM3 proteins with broad-spectrum antiviral function are conserved in swine

306 and Chiroptera, hosts of numerous zoonotic viruses.

307

308 The RACE reactions used here capture multiple possible expressed IFITM paralogues by

309 using primers against the conserved CD225 domain. We assigned microbat IFITM3 using

310 several criteria. First, it encodes a double phenylalanine motif (F8/F9) found only in other

311 IFITM3 orthologues (whereas the other microbat IFITM variants resembled human IFITM2 in

312 having a single phenylalanine at this position). Secondly, microbat IFITM3 had an

313 intracellular location and co-localised with endosomal markers. Conserved genome synteny

314 was previously used to help assign IFITM genes (Smith *et al.*, 2013; Zhang *et al.*, 2012), but

315 was of limited use in the case of the poorly assembled IFITM loci in the pig and microbat

316 genomes. Moreover, the IFITM gene family is associated with numerous processed

317 pseudogenes, gene duplications and copy number variation (Siegrist *et al.*, 2011; Zhang *et*

318 *al.*, 2012), which significantly complicates the assignment of gene orthology. Although, a

319 recent computational study identified 8 pig IFITM family members with expressed sequence

320 tag (EST) evidence (Miller *et al.*, 2014), it lacked the functional validation presented here.

321

322 Functionally important amino acid residues for human or mouse IFITM3 were conserved in

323 pig and microbat IFITM3, indicating that these may be functionally important sites across the

324 orthologues. Amino acid residues are less conserved within IM2 compared to IM1, although

325 IM2 can function as a signal anchor for membrane localisation (Bailey *et al.*, 2013) and is

326 also sufficient to mediate the IFITM3-VAPA interaction (Amini-Bavil-Olyaee *et al.*, 2013).

327

328 We show here that C-terminally HA-tagged IFITM3 (of human, pig and microbat) was clearly

329 detectable at the plasma membrane after live cell staining. These data support recent

330 evidence for a luminal (i.e. extracellular) exposure of the C-terminus (Bailey *et al.*, 2013),

331 and indicate that pig and microbat IFITM3 adopt a similar topology. Other studies have also

332 reported that a proportion of human IFITM3 localises to the plasma membrane (Amini-Bavil-

333 Olyaee *et al.*, 2013; Bailey *et al.*, 2013; Brass *et al.*, 2009), and it is thought that the 20-

334 YEML-23 motif acts as a lysosomal sorting signal for the internalisation of IFITM3 into the

335 endosomal pathway (Jia *et al.*, 2012; John *et al.*, 2013). Pig and microbat IFITM3 contain

336 20-YEML-23 and 20-YEVL-23 respectively (which conform to the consensus sequence for a

337 tyrosine-based sorting signal YxxΦ, where Φ is a residue with a bulky hydrophobic side

338 chain (Bonifacino & Traub, 2003)), and likewise were observed to traffic into endosomes

339 following their cell surface staining. Pig and microbat IFITM3 co-localised with endocytosed

340 transferrin (early endosomes) and CD63 (late endosomes/ MVBs) as seen for human

341 IFITM3 (Figs. 2 and 3 and (Amini-Bavil-Olyaee *et al.*, 2013; Feeley *et al.*, 2011; Huang *et al.*,

342 2011; Jia *et al.*, 2012; Lu *et al.*, 2011). Expression of both microbat and human IFITM3

343 caused expansion of CD63 positive endosomal compartments, consistent with the

344 documented ability of IFITM3 to induce MVB formation (Amini-Bavil-Olyaee *et al.*, 2013).

345 Furthermore, in some cells microbat IFITM3 co-stained with CD63 in ring-like structures, a

346 phenomenon reported for human IFITM3 and enhanced by overexpression of its interaction

347 partner VAPA (Amini-Bavil-Olyaee *et al.*, 2013). In our hands neither human IFITM3 nor its

348 pig and microbat orthologues co-localised with the lysosomal marker LAMP1, which is

349 consistent with some (Yount *et al.*, 2010) but not other (Feeley *et al.*, 2011; Huang *et al.*,

350 2011) reports for IFITM3 localisation. These discrepancies regarding localisation may be

351 due to IFITM3's multiple post-translational modifications (Yount *et al.*, 2012) and/or cell type-

352 dependent differences in its topology (Bailey *et al.*, 2013).

353

354 We show that both pig and microbat IFITM3 restrict cell entry mediated by multiple influenza

355 A virus HA (a class I fusion protein) and lyssavirus G proteins (a class III fusion protein).

356 Restriction at the level of cell entry correlated with significant inhibition of influenza virus

357 yields and NP expression. The microbat, pig and human IFITM3-expressing A549 cells

358 varied in their IFITM3 expression levels, which may underlie variation seen in the degree of

359 restriction. However, anti-influenza restriction by pig IFITM3 was in general lower than that

360 seen for human IFITM3, despite comparable expression levels. We found that pig and

361 human IFITM3 can restrict avian, swine and human influenza A subtypes. Similarly, chicken

362 IFITM3 inhibited viral pseudotypes bearing HAs from both avian and human strains (Smith *et*

363 *al.*, 2013). siRNA knockdown of endogenous pig and microbat IFITM3 enhanced influenza

364 replication by a similar degree to that seen following knockdown of human IFITM3 (Huang *et*

365 *al.*, 2011) or chicken IFITM3 (Smith *et al.*, 2013). IFITM3 was constitutively expressed in pig

366 and bat cells (as reported for other IFITM3 orthologues (Bailey *et al.*, 2012; Everitt *et al.*,

367 2013; Everitt *et al.*, 2012; Friedman *et al.*, 1984; Smith *et al.*, 2013)). Baseline levels of

368 IFITM3 in the pig and bat cells were sufficient to limit viral replication and, following siRNA

369 targeting of baseline IFITM3, microbat IFITM3 was also capable of restricting influenza virus

370 when overexpressed in microbat cells. Since pig IFITM3 was moderately induced by polyI:C

371 and up-regulated upon viral challenge *in vivo* (Andersson *et al.*, 2011; Miller *et al.*, 2014), pig

372 IFITM3 is likely to be relevant to host antiviral responses.

373

374 Here, we show that influenza A viruses and lysssaviruses, virus families which share an

375 ancient co-evolutionary history with bats (Badrane & Tordo, 2001; Tong *et al.*, 2013) are

376 restricted by microbat IFITM3. Bats harbour many diverse virus types and are important

377 reservoirs of zoonotic infections (Drexler *et al.*, 2014; Quan *et al.*, 2013; Smith & Wang,

378 2013; Tong *et al.*, 2013). However, the basis for the intimate association between bats and

379 viruses remains enigmatic, particularly the relative importance of immunological compared to

380 ecological or life history factors (Kupferschmidt, 2013). Although transcriptional induction of

381 chiropteran ISG orthologues is reported (Papenfuss *et al.*, 2012; Zhou *et al.*, 2013), there is

382 a striking lack of functional data for any of these genes. We demonstrate that bats do

383 encode functional IFITM3 and therefore are likely to be competent in this aspect of intrinsic

384 antiviral restriction.

385

386 **Methods**

387

388 **Cells**

389 A549 cells were maintained in F12 medium, and 293T, NPTr cells (Ferrari *et al.*, 2003) and

390 FLN-R cells (Cat No. CCLV-RIE 1091 (Friedrich-Loeffler-Institut, Greifswald-Insel Riems,

391 Germany) were grown in DMEM with 10% FCS. Primary lung cells from the microbat *Myotis*

392 *myotis* were grown in DMEM containing 10% FCS, penicillin and streptomycin and 20%

393 amnioMAX (Gibco).

394

395 **RACE & IFITM3 cloning**

396

397 NPTr and microbat cells were transfected with polyI:C (Invivogen, tlrl-pic) at 33ug/ml using

398 Lipofectamine 2000 and 4h later total RNA and genomic DNA were isolated (Qiagen AllPrep

399 Kit). 5' and 3' IFITM cDNA fragments were generated using the SMARTer RACE cDNA

400 amplification kit (Clontech) and the following primers (designated by name): '5Bat':

401 gcccagagctatgcgtccaccgccaagtgcc; '3Bat': cgtctggtccctgttcaacaccctcttc; '5Pig':

402 gatgttcaggcacttggcggtggaggcatagctc; '3Pig': tgaactggtgctgcctgggcttcgtgg. PCR products

403 were TA cloned and multiple clones sequenced using Sanger sequencing. Non-coding

404 sequences identified by RACE were used to design primers for PCR amplification of full

405 length IFITM3 ('BatIFITM3ncrF': gcatccacacgccatctgctc; 'BatIFITM3ncrR':

406   gaacgccattgtgcacatgtgc; 'PigIFITM3ncrF': acagcttctcctgggcaccatg; 'PigIFITM3ncrR':

407   gtatgtgctgctgtgaaaggag). Pig IFITM3 and microbat IFITM3 were synthesized as codon-

408   optimised genes for expression in human cells (GeneArt) and cloned into the BamHI and

409   NotI sites of the lentivirus vector pSIN-BNHA (derived from pHRSIN-CSGW (Demaison *et*

410   *al.*, 2002)).

411

412   **Creation of IFITM3-expressing cell lines**

413   Lentiviruses expressing IFITM3 (or GFP as a control) were produced by co-transfecting

414   293T cells using Fugene HD with the packaging plasmid p8.91 (Zufferey *et al.*, 1997) (1ug),

415   the VSV-G expressing plasmid pMDG (1ug) and the lentivirus vector pSIN-BNHA (1.5ug)

416   containing either pig IFITM3, microbat IFITM3 or GFP. Supernatants were harvested at 48h

417   and 72h, filtered (0.45um) and used to transduce A549 cells. Transduction efficiency was

418   checked after 48h via flow cytometric detection of HA. Single cell clones were generated

419   using limiting dilution and analysed for HA expression. Those with the most similar

420   expression levels were selected for experiments. Human IFITM3-expressing A549 cells

421   were generated using the same method (Smith *et al.*, 2013).

422

423   **Pseudotyped lentivirus cell entry assay**

424   Lentiviral pseudotypes were produced as previously (Ferrara *et al.*, 2012; Wright *et al.*,

425   2008). For the entry assay, A549 cells were seeded in white 96 well plates (1e4 cells/ well)

426   one day prior to infection with luciferase-expressing pseudotypes expressing influenza

427   hemagglutinin (HA) (H1: GenBank accession AAD17229.1; H5: ABP51969.1; H10:

428   ABI84534.1; H14: BAF43460.1; H7: CAD37074.1; H3: AAA43099.1; H15: AAA96134.1), the

429   G proteins from Mokola virus (MOKV.98/071 RA361: GQ500108), Lagos Bat Virus

430   (LBV.NIG56-RV1: HM623779), West Caucasian Bat Virus (WCBV: AAR03484) or Evelyn

431   Rokitniki Abelseth rabies strain (ERA: ABN11294) or MLV-A or GALV envelope proteins.

432   The H1 and H3 HAs were human in origin, and the other HA subtypes were avian in origin.

433   Cells were infected in triplicate and 48h later luciferase activity was measured using the

434   Bright Glo luciferase assay system (Promega).

435

436   **siRNA transfection and Real-Time PCR**

437   siRNAs against pig IFITM3 (GCTCATAAAGGATTACAGA) or microbat IFITM3

438   (CGAGGACGACGGTGGTCAA) (Dharmacon, ON-TARGETplus) were designed using

439   knowledge of other IFITM paralogues and the Dharmacon siDesign Centre. The ON-

440   TARGETplus non-targeting siRNA pool and an siRNA targeting GFP (P-002048-01-20) were

441   used as controls. siRNA was transfected into cells (20pmol per well of a 12 well plate) using

442   Lipofectamine RNAiMAX. 48h later, RNA was extracted (RNeasy kit, Qiagen) and the

443   Quantitect multiplex RT-PCR kit (Qiagen) was used to measure IFITM3 and GAPDH

444   simultaneously using TaqMan gene expression assays (Applied biosystems, primer

445   sequences available on request). MxPro software was used for comparative quantitation of

446   IFITM3 relative to the GAPDH reference gene.

447

448   **Flow cytometry**

449   Cells were infected in triplicate with influenza A/WSN/33 in media containing 2% FCS. Cells

450   were trypsinised, fixed using BD Fixation/Permeabilisation solution for 20 min and washed

451   twice in BD Perm/Wash buffer before incubation with FITC-conjugated anti-influenza NP IgG

452   (Abcam ab20921) for 40 min at 4 °C. After staining, cells were washed, resuspended in PBS

453   and analysed using a Becton Dickinson FACSCalibur and Cell Quest Pro. For each sample,

454   10,000 cells (gated by forward and side scatter) were analysed for FITC fluorescence.

455

456   **Western blotting**

457   After cell lysis using RIPA buffer, proteins were separated by SDS-PAGE (4-12% TGX gel)

458   and transferred to nitrocellulose membrane. After blocking (5% Marvel, 0.1% Tween-20 in

459   PBS), membranes were incubated for 1h at room temperature with antibodies against

460   influenza NP (Abcam clone C43), beta-actin (Abcam ab8227) or the HA tag (Abcam clone

461   HA.C5). HRP-conjugated secondary antibodies were used followed by enhanced

462   chemiluminescent detection.

463

464   **Virus yield assay**

465   Cells were infected in triplicate with influenza A/WSN/33 in media containing 2% FCS. After

466   1h infection at 37°C, cells were washed thrice in PBS. At the indicated times post infection,

467   supernatants were harvested and the virus yields titered on MDCK cells by plaque assay

468   (Matrosovich *et al.*, 2006).

469

470   **Immunofluorescence**

471

472   Cells were fixed with 4% paraformaldehyde (20 min at room temperature). For transferrin co-

473   localisation, cells were pre-incubated with Alexa 546-conjugated transferrin (Molecular

474   Probes, 5ug/ml) for 10 min before fixation. Cells were permeabilised using 0.2% Triton X-

475   100, blocked using antibody buffer (0.1% Tween 20 and 10% goat serum in PBS) and

476   stained with FITC-conjugated anti-HA (Bethyl Laboratories A190-108F), anti-LAMP1 (Abcam

477   clone H4A3) or anti-CD63 (Santa Cruz MX-49.129.5) for 1 h at room temperature. Dylight

478   594-conjugated goat anti-mouse IgG was used to detect anti-CD63 and anti-LAMP1

479   antibodies. Live cell staining was performed either at 37°C or on ice, with solutions

480   equilibrated accordingly. Cells were washed twice in serum-free media before incubation for

481   30 min with FITC conjugated anti-HA and Alexa 546-conjugated transferrin (in serum free

482   media). Cells were then quickly washed in PBS and immediately fixed using 4%

483   paraformaldehyde (20 min). Coverslips were mounted using ProLong Gold reagent with

484   DAPI and examined with a Zeiss LSM 780 confocal microscope.

485

486   **Sequence analysis**

487   Multiple sequence alignments were performed using Clustal O (1.2.0) and Seaview (Gouy *et*

488   *al.*, 2010) was used for manual editing.

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Figure Legends

726   **Figure 1. Multiple sequence alignments with microbat and pig IFITM3.** (**a**) Pig and

727   microbat IFITM3 nucleotide sequences were aligned with experimentally verified IFITM3

728   orthologues. The intron-exon boundary is shown (exon1: black, exon 2: blue) and intronic

729   sequences flanking the splice sites are in red lower case. * denotes identical nucleotides. (**b**)

730   Pig and microbat IFITM3 amino acid sequences were aligned with other IFITM proteins. '*****'

731   indicates identical amino acid residues, ':' indicates residues with strongly similar properties,

732   '.' indicates residues with weakly similar properties. The arrow indicates the exon-exon

733   boundary. Protein domains of human IFITM3 are shown (according to (John *et al.*, 2013))

734   (intramembrane domain 1 (IM1), conserved intracellular loop (CIL) and intramembrane

735   domain 2 (IM2)). Highlighted residues are discussed within the text.

736

737   **Figure 2. Subcellular localization of IFITM3 proteins.** A549 cells stably expressing HA-

738   tagged IFITM3 proteins from human, pig or microbat (or untransduced cells) were fixed,

739   permeabilised and stained for HA, CD63 (**b**) or LAMP1 (**c**). In (**a**) cells were incubated with

740   Alexa 546-conjugated transferrin before fixation and staining with FITC-conjugated anti-HA.

741   Nuclei were stained with DAPI and coverslips examined by confocal microscopy. Images

742   show representative staining patterns. Scale bar measures 10 um. Arrows in (**b**) identify

743   'ring-like' staining of microbat IFITM3-HA and CD63.

744

745   **Figure 3. Endocytic uptake of IFITM3 proteins**. Live A549 cells expressing HA-tagged

746   IFITM3 from human, pig or microbat (or untransduced cells) were incubated either on ice (**a**)

747   or at 37°C (**b**) with FITC-conjuated anti-HA and Alexa 546-conjugated transferrin. After cell

748   fixation, nuclei were stained with DAPI, and coverslips examined by confocal microscopy.

749   Scale bar measures 10 um.

750

751   **Figure 4. Microbat and pig IFITM3 inhibit influenza HA-mediated cell**

752 **entry**. A549 cells stably expressing IFITM3 from human, pig (**a**) or microbat (**b**)

753 (or untransduced cells) were infected with pseudotyped viruses which express different

754 influenza HA glycoproteins. 48h post-infection, luciferase reporter activity was measured and

755 normalized to untransduced cells (relative infectivity of 1 corresponds to 3,000-5,000 relative

756 light units according to pseudotype used). Mean+ SD is shown (n=3) and data is

757 representative of 3 independent experiments.

758

759 **Figure 5. Microbat and pig IFITM3 inhibit influenza virus replication**. A549 cells stably

760 expressing human, pig or microbat IFITM3 were infected with influenza A virus. In (**b**)-(**d**) pig

761 IFITM3 clone 1 and microbat IFITM3 clone 2 were used. (**a**) 7h post-infection with influenza

762 A/WSN/33 NP expression was analysed by flow cytometry. (**b**) 16h after infection with

763 influenza A/WSN/33 or A/swine/453/06 or mock-infection (-V) cells were lysed and Western

764 blotting performed to detect influenza NP, actin and HA-tagged IFITM3 or GFP. Molecular

765 weight (MW) of protein size markers is indicated. Virus yields following infection with

766 influenza A/WSN/33 at m.o.i. 3 (**c**) or m.o.i. 0.01 (**d**) were measured using plaque assays.

767 Mean+ SD is shown (n=3). *: $p<0.05$, **: $p<0.01$ and ****:$p<0.0001$ relative to GFP-

768 expressing cells (Student's *t* test).

769

770 **Figure 6. Microbat and pig IFITM3 inhibit cell entry mediated by lyssavirus G proteins**.

771 A549 cells stably expressing either GFP, human IFITM3, pig IFITM3 (clone 1) or microbat

772 IFITM3 (clone 2) were infected with pseudotyped viruses which express the envelope

773 glycoprotein from MOKV (Mokola virus), WCBV (West Caucasian Bat Virus), LBV (Lagos

774 Bat Virus) or ERA (Evelyn Rokitniki Abelseth rabies strain). 48h post-infection, luciferase

775 reporter activity was measured and normalized to GFP-expressing cells (relative infectivity of

776 1 corresponds to 25,000-80,000 relative light units according to pseudotype used). Mean+

777 SD is shown (n=3) and data is representative of 3 independent experiments.

778

779 **Figure 7. Microbat and pig IFITM3 are polyI:C responsive**. Pig NPTr (**a**) and microbat (**b**)

780 cells were either mock-treated or stimulated by addition of polyI:C to cell media (50ug/ml) or

781 transfection of polyI:C (33ug/ml) using Lipofectamine 2000 (microbat cells only). 7h later

782 qRT-PCR was used to quantitate IFITM3 and the reference gene GAPDH. Fold change in

783 IFITM3 mRNA is expressed relative to mock-treated cells. Mean+ SD shown for biological

784 triplicates assayed in duplicate.

785

786 **Figure 8. siRNA knockdown of IFITM3 in pig and microbat cells enhances influenza**

787 **virus replication**. Pig NPTr or microbat cells were transfected with siRNA targeting IFITM3

788 or control siRNA prior to quantitation of IFITM3 by qRT-PCR (**a** & **e**) or infection with

789 influenza A/WSN/33. Virus yields were measured by plaque assay (**b**, **f**) or NP expression

790 was measured using flow cytometry (**c, d, g**). NPTr cells were either stimulated with polyI:C

791 for 2h before infection (m.o.i. 0.01) and analysed 36 h post-infection (**b** & **c**) or otherwise

792 cells were infected in the absence of polyI:C. Mean+ SD shown for biological triplicates

793 assayed in duplicate and data is representative of 2 independent experiments. *: $p<0.05$ and

794 **: $p<0.01$ (Student's *t* test).

795

796

797

798

(a)

Exon 1

```
CHICKEN_IFITM3   --------------ATGGAGCGGGTACGCGCTTCGGGTCCGGGAGTCCCACCG---TAT 42
MOUSE_IFITM3     ATGAACCACACTTCTCAAGCCTTCATCACCGCTGCCAGTGGAGGACAGCCCCCAAACTAC 60
MICROBAT_IFITM3  ATGAACCCCAACTCCCAGCCCTTCTTCTCTGGCGCCCGCGCCGGAGTGTCCCCATCCTAT 60
PIG_IFITM3       ATGAACTGCGCTTCCCAGCCCTTCTTCACTGGTGCCCATGGAGGA---CCCCAACATAT 57
HUMAN_IFITM3     ATGAATCACACTGTCCAAACCTTCTTCTCTCCTGTCAACAGTGGCCAGCCCCCCAACTAT 60
                                *        **     *  **    * **    **

CHICKEN_IFITM3   GAACCCCTGATGGACGGGATGGAC------ATGGAGGGGAAG------------------ 78
MOUSE_IFITM3     GAAAGAATCAAGGAAGAATATGAGGTGGCTGAGATGGGGGCACCGCACGGATCGGCTTCT 120
MICROBAT_IFITM3  GAGGTGCTCAAGGAGGAACACGAGGTGTCTGTGCTGGGGGGTCCCCAGAGCTCGATGGCA 120
PIG_IFITM3       GAGATGCTCAAGGAGGAGCACGAGGTGGCCGTGCTGGGGGGTCCCCAGACCTCAGCCCCC 117
HUMAN_IFITM3     GAGATGCTCAAGGAGGAGCACGAGGTGGCTGTGCTGGGGGGGCGCCCCACAACCCTGCTCCC 120
                 **      *  * *** *   **       *  ****

CHICKEN_IFITM3   ACCCGCAGCACGGTGGTGACGGTG------GAGACGCCCCTGGTGCCTCCTCCCCGCGAC 132
MOUSE_IFITM3     GTCAGAACTACTGTGATCAACATGCCCAGAGAGGTGTCGGTG------------CCTGAC 168
MICROBAT_IFITM3  GCGAGGACGACGGTGGTCAACATCCAGACTGACACAGTGGTG------------CCCGAC 168
PIG_IFITM3       GTGGCCACCACGGTGATCAACATCCGAAGCGAGACCTCCGTG------------CCCGAC 165
HUMAN_IFITM3     CCGACGTCCACCGTGATCCACATCCGCAGCGAGACCTCCGTG------------CCCGAC 168
                 **  *** *  * *         **    *  **   **    *  ***

CHICKEN_IFITM3   CACCTGGCCTGGTCGCTGTGCACCACGCTGTACGCCAACGTCTGCTGCCTCGGCTTCCTG 192
MOUSE_IFITM3     CATGTGGTCTGGTCCCTGTTCAATACACTCTTCATGAACTTCTGCTGCCTGGGCTTCATA 228
MICROBAT_IFITM3  CACATCGTCTGGTCCCTGTTCAACACCCTCTTCTTCAACCCCTGCTGCCTGGGCTTCGTG 228
PIG_IFITM3       CACGTCGTCTGGTCCCTGTTCAACACCCTCTTCATGAACTGGTGCTGCCTGGGCTTCGTG 225
HUMAN_IFITM3     CATGTCGTCTGGTCCCTGTTCAACACCCTCTTCATGAACCCCTGCTGCCTGGGCTTCATA 228
                 **  * * ***** ****  **  ** ** *   ***  ********* ****** *
```

Intron Exon 2

```
CHICKEN_IFITM3   GCGCTCGTCTTCTCCGTGAAGgttggggaat...gtctccccagTCCAGGGATCGCAAAGT 230
MOUSE_IFITM3     GCCTATGCCTACTCCGTGAAGgtgagtgtgc...tctgctccagTCTAGGGATCGGAAGAT 266
MICROBAT_IFITM3  GCGTTCGCCTACTCCGTGAAGgtgggtgcgc...tctccccagTCTAGGGACCGGAAGAT 266
PIG_IFITM3       GCTTTCGCCTACTCCGTGAAGgtgggtgggc...tctcctccagCGGAGGGACCGGAAGAT 263
HUMAN_IFITM3     GCATTCGCCTACTCCGTGAAGgtgcgtatgg...tctccccagTCTAGGGACAGGAAGAT 266
                 **   * ** **********          * ***** * **  *

CHICKEN_IFITM3   CCTGGGTGACTACAGCGGGGCGCTCAGCTATGGCTCCACTGCGAAGTACCTGAACATCAC 290
MOUSE_IFITM3     GGTGGGTGATGTGACTGGAGCCCAGGCCTACGCCTCCACTGCTAAGTGCCTGAACATCAG 326
MICROBAT_IFITM3  GGTGGGAGACGTGATTGGGGCCCAGAGCTATGCGTCCACCGCCAAGTGCCTGAACATCTG 326
PIG_IFITM3       GGTGGGAGACATCATTGGGGCCCAGAGCTATGCCTCCACCGCCAAGTGCCTGAACATCTG 323
HUMAN_IFITM3     GGTTGGCGACGTGACCGGGGCCCAGGCCTATGCCTCCACCGCCAAGTGCCTGAACATCTG 326
                  * ** **      *  ** ** *    *** *  ***** ** ****  **********

CHICKEN_IFITM3   GGCCCATCTGATCAACGTCTTCCTCATCATCCTCATCATCGCCCTGGTTGCCAGTGGCAC 350
MOUSE_IFITM3     CACCTTGGTCCTCAGCATCCTGATGGTTGTTATCACCATTGTTAGTGTC----------- 375
MICROBAT_IFITM3  GGCCGTGGTCTTGGGCCTCCTTGTCATAGTTGCCGGTCATCATCGCCGGTC----------- 375
PIG_IFITM3       GGCTCTGGTCCTGGGCCTCCTTCTGATCATTGCGTTCATCATCGTTTGCACCACTGGCTC 383
HUMAN_IFITM3     GGCCCTGATTCTGGGCATCCTCATGACCATTCTGCTCATCGTCATCCCA----------- 375
                  *    * *   * ** *  *    *     * *   ***

CHICKEN_IFITM3   CATCATGGTGGCCAACATCTTCAACCACCAGCAGCAACACCCCGAATTCATTGGACCCAC 410
MOUSE_IFITM3     -ATCATCATTGTTCTTAACGCTCAAAACCTTCACACTTAA------------------- 414
MICROBAT_IFITM3  -GTCGTATTTTACACCTCTGCACTGAAT------CGGGTACGTGGGTTCCTATCACCT 428
PIG_IFITM3       CCTGGTAATTTTCCCAAGCAGTTCTGCAGCTCATAAAGGATTACAGAGGCTACTAG----- 438
HUMAN_IFITM3     -GTGCTGATCTTCCAGGCCTATGGATAG------------------------------ 402
                  *  *  *                   *

CHICKEN_IFITM3   TTAG---------- 414
MOUSE_IFITM3     -------------
MICROBAT_IFITM3  GCCCCAAGACTGA 441
PIG_IFITM3       -------------
HUMAN_IFITM3     -------------
```

(b)

```
HUMAN_IFITM1      ---------------------MHKEEHEVAVLGPPPSTILPRSTVINIHSETSV----PDHV  37
HUMAN_IFITM2      MNHIVQT-FSPVNSGQPPNYEMLKEEQEVAMLGVPHNPAPPMSTVIHIRSETSV----PDHV  57
CHICKEN_IFITM3    -----MERVRASGPGVPP-YEPLMDGMD--MEGK------TRSTVVTV--ETPLVPPPRDHL  46
CHIMPANZEE_IFITM3 MNHTVQTFFSPVNSGQPPNYEMLKEEHEVAVLGAPHNPAPPMSTVIHIRSETSV----PDHV  58
MOUSE_IFITM3      MNHTSQAFITAASGGQPPNYERIKEEYEVAEMGAPHGSASVRTTVINMPREVSV----PDHV  58
MICROBAT_IFITM3   MNPNSQPFFSGARAGVSPSYEVLKEEHEVSVLGGPQSSMAARTTVVNIQTDTVV----PDHI  58
PIG_IFITM3        MNCASQPFFTGAHGG-PPTYEMLKEEHEVAVLGAPQTSAPVATTVINIRSETSV----PDHV  57
HUMAN_IFITM3      MNHTVQTFFSPVNSGQPPNYEMLKEEHEVAVLGAPHNPAPPTSTVIHIRSETSV----PDHV  58
                                     :  :   *         :**:: :  :           **:
```

N-terminus

```
HUMAN_IFITM1      VWSLFNTLFLNWCCLGFIAFAYSVKSRDRKMVGDVTGAQAYASTAKCLNIWALILGILMT  97
HUMAN_IFITM2      VWSLFNTLFMNTCCLGFIAFAYSVKSRDRKMVGDVTGAQAYASTAKCLNIWALILGIFMT 117
CHICKEN_IFITM3    AWSLCTTLYANVCCLGFLALVFSVKSRDRKVLGDYSGALSYGSTAKYLNITAHLINVFLI 106
CHIMPANZEE_IFITM3 VWSLFNTLFMNPCCLGFIAFAYSVKSRDRKMVGDVTGAQAYASTAKCLNIWALILGILMT 118
MOUSE_IFITM3      VWSLFNTLFMNFCCLGFIAYAYSVKSRDRKMVGDVTGAQAYASTAKCLNISTLVLSILMV 118
MICROBAT_IFITM3   VWSLFNTLFFNPCCLGFVAFAYSVKSRDRKMVGDVIGAQSYASTAKCLNIWAVVLGLLVI 118
PIG_IFITM3        VWSLFNTLFMNWCCLGFVAFAYSVKARDRKMVGDIIGAQSYASTAKCLNIVALVLGLLLI 117
HUMAN_IFITM3      VWSLFNTLFMNPCCLGFIAFAYSVKSRDRKMVGDVTGAQAYASTAKCLNIWALILGILMT 118
                  .*** .**: * *****:*  .:***:****::** ** :*.**** *** : :: :::
```

IM1   CIL   IM2

CD225 domain

```
HUMAN_IFITM1      IGFILLLVFGSVTVYHIMLQIIQEKRGY------ 125
HUMAN_IFITM2      ILLIIIP----VLVVQAQR--------------- 132
CHICKEN_IFITM3    ILIIALVASGTIMVANIFNHQQQHPEFIGPT--- 137
CHIMPANZEE_IFITM3 ILLIVIP----VLIFQAYG--------------- 133
MOUSE_IFITM3      VITIVSV----IIIVLNAQNLHT----------- 137
MICROBAT_IFITM3   VAVIIAV----VVFYTSALN--RGYVGSYHLPQD 146
PIG_IFITM3        IAFIIVCTTGSLVIFQAVLQLIKDYRGY------ 145
HUMAN_IFITM3      ILLIVIP----VLIFQAYG--------------- 133
                  :  *       :  . 
```

C-terminus
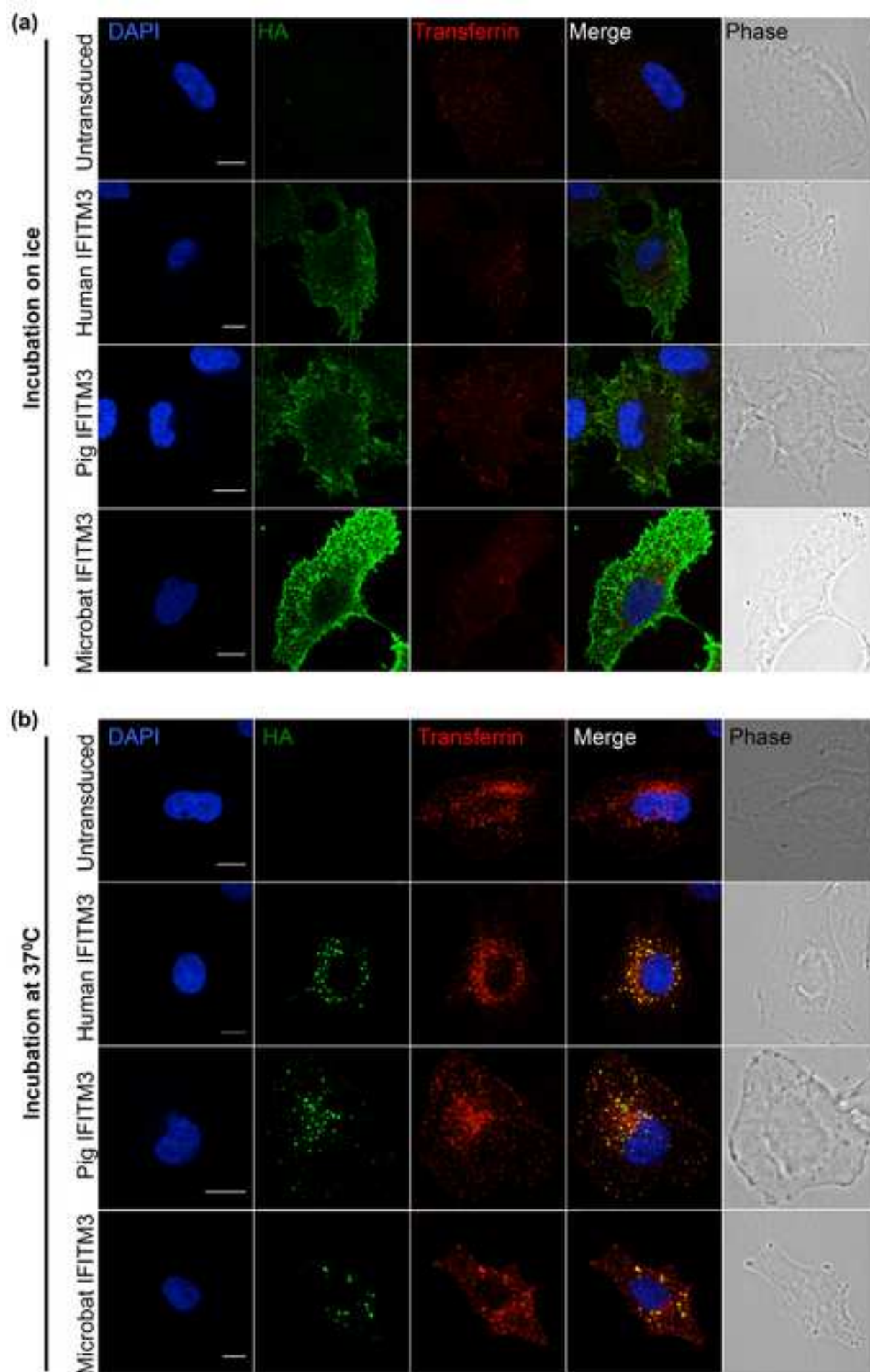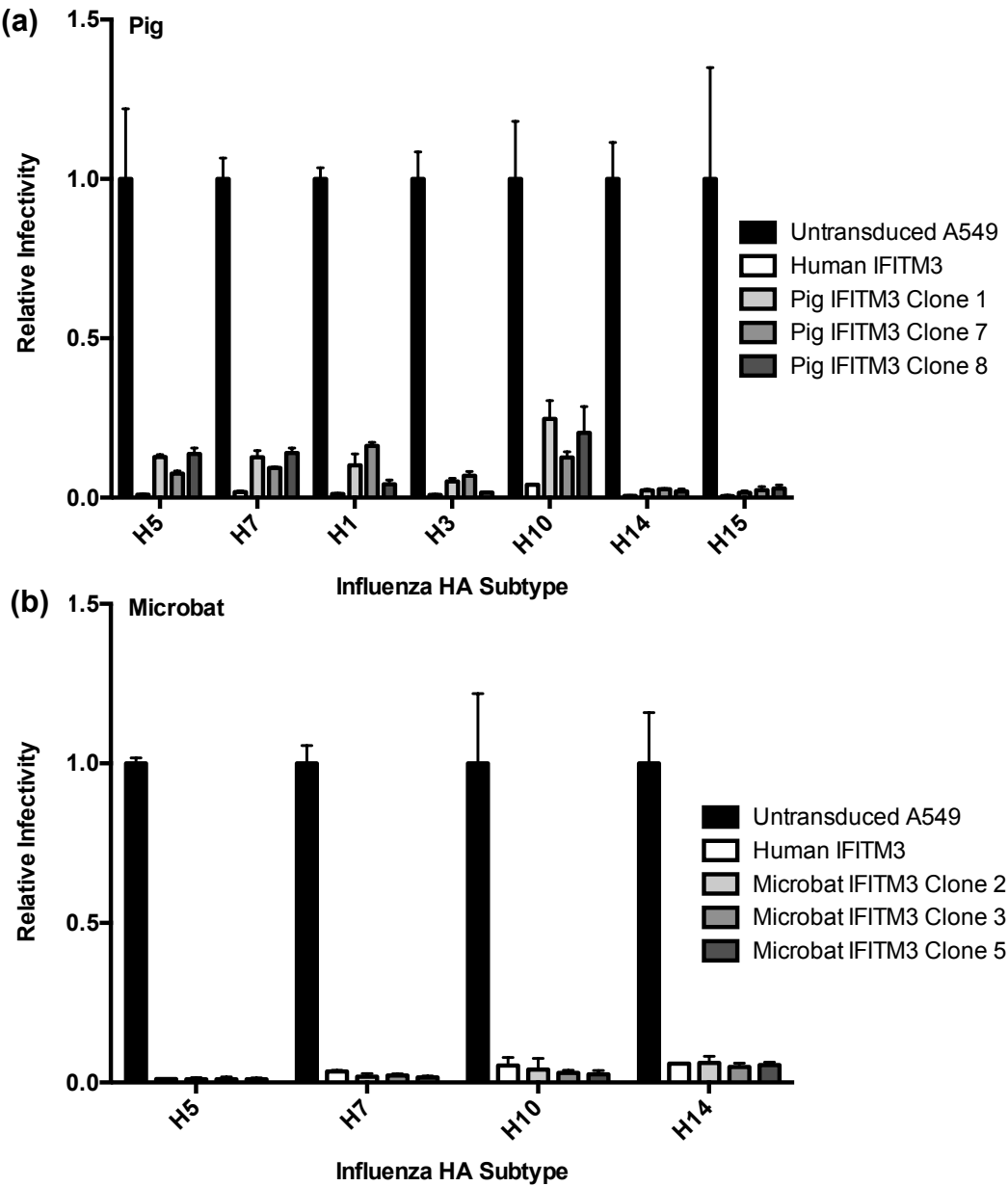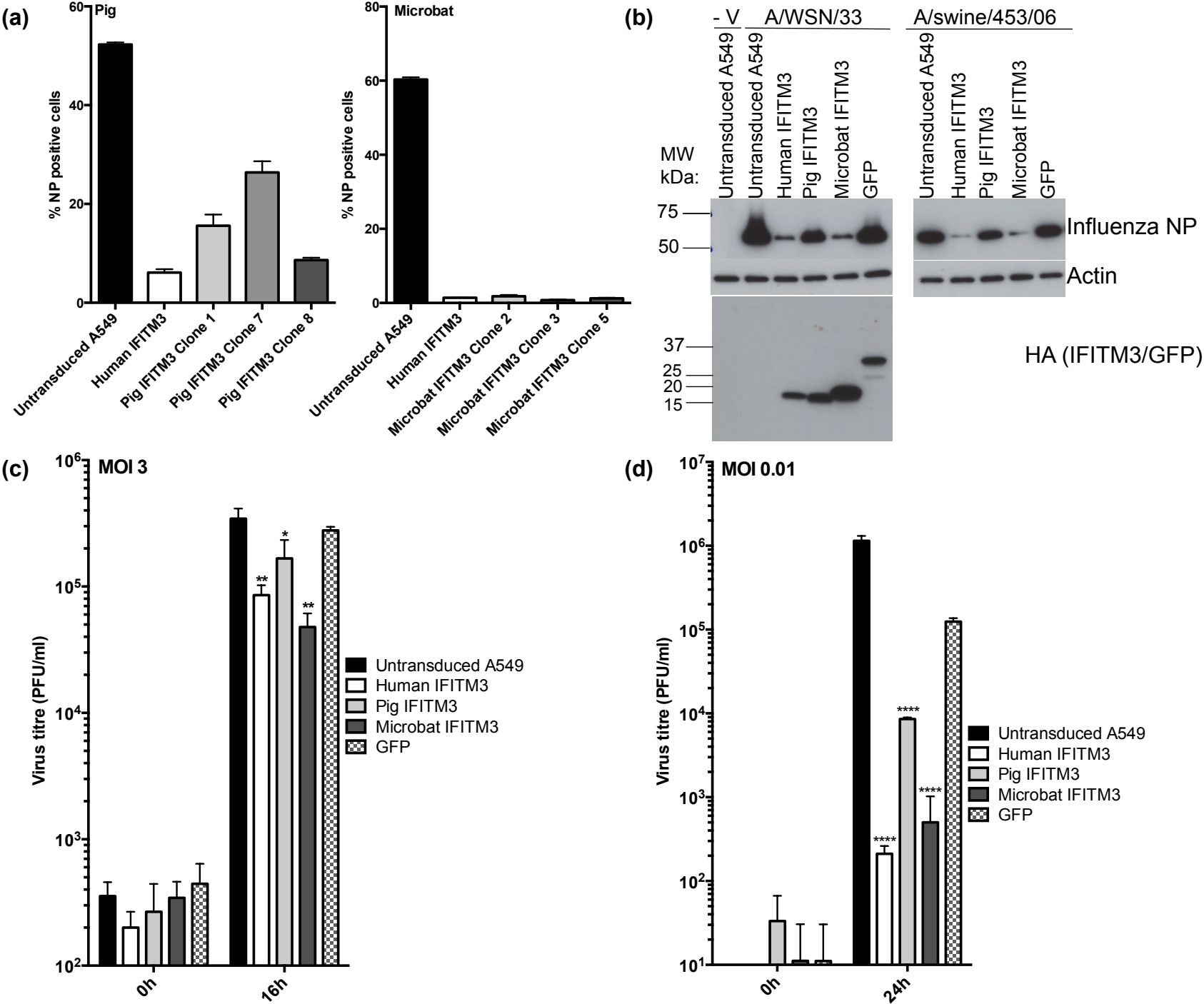
Figure3

Figure5
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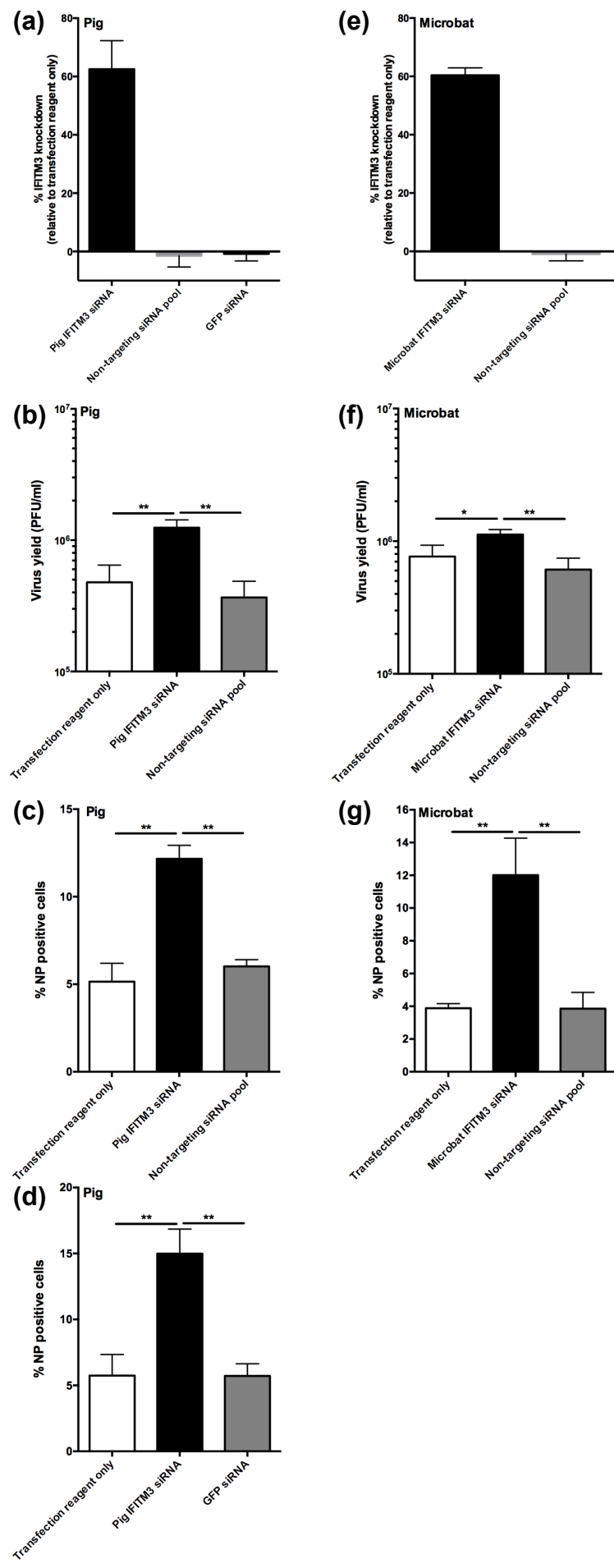
Figure7
Click here to download Figure: FIGURE7.pdf

(a)

Pig

Fold change in IFITM3 mRNA

Mock treated

Poly I:C

(b)

Microbat

Fold change in IFITM3 mRNA

Mock treated

Poly I:C addition

Poly I:C transfection

Figure8

Supplementary Material Files