Human IFIT1 inhibits mRNA translation of rubulaviruses but not other members of the Paramyxoviridae family.

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Running title: IFIT1 inhibition of paramyxoviruses

Key words: Paramyxoviruses, interferon, IFIT1

Abstract

We have previously shown that IFIT1 is primarily responsible for the antiviral action of interferon (IFN) alpha/beta against parainfluenza virus (PIV) type 5, selectively inhibiting the translation of PIV5 mRNAs. Here we report that whilst PIV2, PIV5 and mumps virus (MuV) are sensitive to IFIT1, non-rubulavirus members of the paramyxoviridae such as PIV3, Sendai virus (SeV) and canine distemper virus (CDV) are resistant. The IFIT1-sensitivity of PIV5 was not rescued by co-infection with an IFIT1-resistant virus (PIV3), demonstrating that PIV3 does not specifically inhibit the antiviral activity of IFIT1 and that the inhibition of PIV5 mRNAs is regulated by cis-acting elements. We developed an *in vitro* translation system using purified human IFIT1 to further investigate the mechanism of action of IFIT1. Whilst the translation of PIV2, PIV5 and MuV mRNAs were directly inhibited by IFIT1, the translation of PIV3, SeV and CDV mRNAs were not. Using purified human mRNA capping enzymes we show biochemically that efficient inhibition by IFIT1 is dependent upon a 5' guanosine nucleoside cap (which need not be N7-methylated) and that this sensitivity is partly abrogated by 2'O methylation of the cap 1 ribose. Intriguingly, PIV5 M mRNA, in contrast to NP mRNA, remained sensitive to inhibition by IFIT1 following *in vitro* 2'O methylation, suggesting that other structural features of mRNAs may influence their sensitivity to IFIT1. Thus, surprisingly, the viral polymerases (which have 2'-O-methyltransferase activity) of rubulaviruses do not protect these viruses from inhibition by IFIT1. Possible biological consequences of this are discussed.

Importance

Paramyxoviruses cause a wide variety of diseases and yet most of their genes encode for structural proteins and proteins involved in their replication cycle. Thus the amount of genetic information that determines the type of disease paramyxoviruses cause is relatively small. One factor that will influence disease outcomes is how they interact with innate host cell defences, including the interferon (IFN) system. Here we show that different paramyxoviruses interact in distinct ways with cells in a pre-existing IFN-induced antiviral state. Strikingly, all the rubulaviruses tested were sensitive to the antiviral action of ISG56/IFIT1, whilst all the other paramyxoviruses tested were resistant. We developed novel *in vitro* biochemical assays to investigate the mechanism of action of IFIT1, demonstrating that the mRNAs of rubulaviruses can be directly inhibited by IFIT1 and that this is at least partially because their mRNAs are not correctly methylated.

Introduction

Paramyxoviruses are a large group of negative-sense single-stranded RNA viruses that cause a wide variety of animal and human diseases. The Paramyxoviridae family is divided into two subfamilies, the paramyxovirinae and the pneumovirinae subfamilies. The paramyxovirinae are further subdivided into a number of genera including morbilliviruses [e.g. measles virus (MeV) and canine distemper virus (CDV)], respiroviruses [e.g. Sendai virus (SeV) and parainfluenza virus type 3 (PIV3)] and rubulaviruses [e.g. mumps virus (MuV), PIV2 and PIV5]. Paramyxoviruses are enveloped viruses, the viral glycoproteins protrude from the outer surface of the envelope and function to attach the viruses to their target cells. On the inner surface of the envelope is the matrix (M) protein, which is required

for the structural integrity of the virion. The envelope surrounds a helical nucleocapsid, in which the nucleocapsid protein (NP) encapsidates genomic or antigenomic RNA. Associated with the nucleocapsid is the virally-encoded polymerase complex. The viral polymerase both transcribes and replicates the viral genome. Viral mRNAs are capped and polyadenylated by the viral polymerase (for reviews of the molecular biology of paramyxoviruses see (1, 2)).

Despite their limited genetic information the majority of paramyxoviruses encode small multifunctional accessory proteins which function to aid virus multiplication and block cellular antiviral defence mechanisms; typically these proteins can block both the production of, and signaling response to, interferons (IFNs) (for reviews see (3-7)). Significantly, the mechanisms of action of these multifunctional IFN antagonists differ from one virus to another. Undoubtedly these properties, and in general how paramyxoviruses interact with the IFN system and other innate defence mechanisms, are likely to be major factors in determining the type of disease each virus causes (8).

The IFN response is an extremely powerful antiviral defence system that, unless counteracted by viruses, will limit their replication to such a degree that they will not cause disease or be efficiently transmitted between susceptible hosts (8, 9). Infected cells detect the presence of viruses due to the production by viruses of molecules with molecular signatures (pathogen associated molecular patterns or PAMPs), such as dsRNA, which activate the IFN-induction cascade and result in the secretion of IFN-α/β from infected cells (9, 10). The release of IFN induces an antiviral state in neighbouring uninfected cells by up-regulating the expression of hundreds of interferon stimulated genes (ISGs), many of which have direct or indirect antiviral activity (11). Most paramyxoviruses counteract the IFN responses by

94 producing proteins that block IFN induction and/or IFN signaling by a variety of
95 mechanisms (3-7). Furthermore, they tightly control viral transcription and replication,
96 thereby limiting the production of PAMPs that may activate the IFN response (12, 13).
97 Indeed, it is probably mistakes that viruses make during transcription and replication, such as
98 the production of copyback defective interfering particles, that activate the IFN response
99 (14-16), reviewed in (17). Nevertheless, the ability of paramyxoviruses to block the IFN
100 response both in tissue culture cells and *in vivo* is not absolute and some IFN-α/β will be
101 produced (18, 19). Furthermore, IFN-γ, which can also induce an antiviral state in cells, will
102 also be produced by activated subsets of lymphocytes (20). Therefore it is inevitable that
103 viruses will infect cells in a pre-existing IFN-induced antiviral state, potentially limiting the
104 speed of virus replication and spread. Although IFNs induce hundreds of ISGs, several ISGs
105 with direct antiviral activity have been shown to be specific for families or groups of related
106 viruses (11, 21, 22). With regard to the paramyxoviridae family we have previously shown
107 that ISG56/IFIT1 (hereinafter referred to as IFIT1), which selectively inhibits translation, is
108 the primary effector of the IFN-induced antiviral state that limits the replication of the
109 rubulavirus PIV5 (23). Pretreatment of cells with IFN-α/β inhibits PIV5 protein synthesis
110 but not cellular protein synthesis. This is because IFIT1 selectively inhibits the translation of
111 PIV5 mRNAs but does not affect cellular mRNAs (23).
112
113 Mammalian mRNAs have N-7 methyl guanosine (m^7GpppN), termed cap 0, at their 5' end
114 that recruits factors involved in RNA processing and translation initiation. The first and
115 second nucleosides of mammalian mRNAs are also methylated on the 2' hydroxyl group of
116 the ribose ring, generating cap 1 and cap 2 respectively. Whilst cap 1 and cap 2 are not

117 required for efficient mRNA translation, IFIT1 can inhibit the translation of mRNAs that
118 lack cap 1 (24-27). IFIT1 also binds uncapped, 5'-triphosphorylated RNA, characteristic of
119 the 5' ends of the genomic and antigenomic RNAs of some RNA viruses, as well as those of
120 some viral transcripts (28); for reviews on the mechanism of action of IFIT1 and the IFIT
121 family of proteins see (21, 26, 27, 29). However, recent evidence suggests that there are
122 differences in the mechanisms of action of the murine and human paralog IFIT1 proteins.
123 Whilst murine IFIT1 (IFIT1B) inhibits the translation of mRNAs that lack cap 1, it has been
124 proposed that human IFIT1 recognises some other, as yet undefined, structure near the cap,
125 or possibly that 5' mRNA sequences may help define the specificity of inhibition by human
126 IFIT1 (30). The RNA-capping activity of viral RNA polymerases often include 2'-O-
127 methyltransferases (2'-O-MTases) which modify cap 1 and thus can avoid inhibition by
128 IFIT1(B), as evidenced by the sensitivity of virus mutants that lack 2'-O-MTase activity (for
129 reviews see (21, 26)). Capping and methylation of viral RNAs are also be important as such
130 modifications can prevent the activation of RIG-I, thereby reducing the amount of IFN
131 produced by virally infected cells (for review see (31)).
132
133 Here we have examined the ability of IFIT1 to inhibit the translation of a variety of
134 paramyxovirus mRNAs, and thus the replication of those viruses. We show that whilst all
135 rubulaviruses tested were sensitive to IFIT1, all non-rubulavirus members of the
136 paramyxoviridae tested were insensitive. Lack of 2' O-methylation of rubulavirus mRNAs
137 was at least partially responsible for their inhibition by IFIT1. The possible biological
138 consequences of differences in sensitivity of paramyxoviruses to IFIT1 are discussed.
139
140 **Methods**

Cells, viruses, antibodies and interferon

A549 cells, and derivatives, were grown as monolayers in 25-cm^2, 75-cm^2, or 300-cm^2 tissue culture flasks in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum at 37^0C. Where appropriate, cells were treated with human recombinant interferon (Intron A, Merck, Sharpe and Dohme) at 1000 units/ml. Viruses used in these studies were; PIV2 (Colindale strain), PIV3 (Washington and JS strains and recombinant ΔC and ΔD JS viruses (32)), PIV5 (formerly known as SV5: strains W3 (33), CPI+ and CPI- (34)), MuV (Enders (35)), RSV (36), Sendai (Cantell strain, free of defective interfering particles), and canine distemper virus (Mill Hill strain). Plaque assays were performed by standard methods in six-well dishes that included 0.1% Avicel (FMC Biopolymer) in the overlay medium. Plaques were visualized by immunostaining by using a pool of monoclonal antibodies or polyclonal antisera specific for the different viruses as described previously (37), together with alkaline phosphatase-conjugated secondary antibody by using SIGMAFAST BCIP/NBT as the substrate.

Preparation of [^{35}S]-L-methionine labeled total-cell extracts and SDS-PAGE.

Infected or uninfected cells that had or had not been pretreated with IFN for 12h prior to infection, were metabolically labeled for 1h with [^{35}S]-L-methionine (500Ci/mmol, MP Biomedical, USA) at 18h p.i. After labeling, cells were lysed in disruption buffer, sonicated and heated for 5 min at 100^0C then analyzed by gel electrophoresis (SDS-PAGE). The gels were fixed, stained, dried and resolved bands visualized by phosphoimager analysis. Where

appropriate the same amount of cell equivalents were run on PAGE. Furthermore the amount of protein in each sample was monitored by staining the polyacrylamide gels with Coomassie Brilliant Blue.

Immunofluorescence

Cells to be stained for immunofluorescence were grown on 10-mm-diameter coverslips (MIC3270, Scientific Laboratory Supplies, UK). Cells were stained with specific mAbs, as described in detail elsewhere (38). Briefly, monolayers were fixed with 5% formaldehyde, 2% sucrose in PBS for 10 min at 20°C, permeabilized with 0.5% Nonidet-P40, 10% sucrose in PBS for 5 min at 20°C, and washed three times in PBS containing 1% calf serum. PIV5- and PIV3- infected cells were detected by indirect immunofluorescence using a secondary goat anti-mouse Ig Texas Red-conjugated antibody (Abcam; catalog number ab6787). The primary antibodies were PIV5-NP-a and PIV5-Pe for PIV5 (39) and 4721, 2281 and 4812 for PIV3 (40). After staining for immunofluorescence the monolayers of cells were examined with the use of a Nikon Microphot-FXA immunofluorescence microscope.

RNA selection and in vitro translation.

RNA for *in vitro* translations was isolated by sedimentation through CsCl gradients by a modified method described by Leppert et al (41). Confluent monolayers of infected cells, grown in 300-cm^2 flasks were resuspended in ice-cold lysis buffer [(150mM NaCl, 50mM Tris-HCl pH 7.5, 0.6% NP-40, protease-inhibitor cocktail (Roche, complete Mini EDTA-free, 1 tablet per 7 ml of buffer)] at 1 - 2 x 10^8 cells per ml and left on ice for 5 minutes prior to vortexing for 2 minutes. Nuclei were removed by centrifugation twice at 4,200 x g for 5 minutes at 4°C. The supernatant (cytoplasmic extract) was collected, made to 6mM EDTA

and layered onto 35% w/w CsCl in 25mM Tris-HCl pH 7.5, 2mM EDTA followed by centrifugation at 175,000 x g, at 12 °C for 16 – 18h. Naked RNA (including mRNA) forms a pellet at the bottom of the gradient, whilst viral genomic and antigenomic RNAs remain complexed with nucleoprotein and do not enter the 35% CsCl cushion. The supernatant was discarded and the pellet resuspended in RNase-free water and adjusted to 1µg/µl. Selected RNA was translated *in vitro* with a rabbit reticulocyte lysate kit (L4960, Promega) in the presence of $^{[35]}$S-methionine/cysteine (Perkin Elmer, NEG772, EasyTag^{TM} Express Protein Labeling Mix) using a modification to the manufacturer's instructions: Methionine/cysteine-free medium (Sigma, D0422) was used to provide other amino acids (1µl per 50µl reaction).

Capping and methylation of mRNA

Human RNA guanylyltransferase and 5'-phosphatase (RNGTT), RNA guanine-7 methyltransferase (RNMT) and cap methyltransferase 1 (CMTR1) were synthesized and purified according to Gonatopoulos-Pournatzis et al (42). As described, the enzymes were all verified as being active by *in vitro* reactions followed by thin layer chromatography. Capping and methylation reactions were carried out in 50mM Tris-HCl, pH 8.0, 6mM KCl, 1.25mM $MgCl_2$, 1mM DTT buffer as follows: 1µl 10x buffer, 1µl RNGTT (2.5 mg/ml), 1µl RNMT (0.5 mg/ml), CMTR1 (0.28 mg/ml), 1µl SAM (2mM), 1µl GTP (1mM), 0.5 µl RNasin, 2µl RNA (1 µg/µl). The reaction mixture was made up to 10µl with H_2O, including experiments in which RNGTT, RNMT or CMTR1 were omitted, and incubated at 37^{o}C for 1h.

Cloning and purification of IFIT1

IFIT1 was amplified with primer IFIT1F/IFIT1Xho from the plasmid pGAC-HA-IFIT1 , restricted with Nco I and Xho I, ligated with a modified pLOU3, in which MBP was replaced with SUMO, while Sal I in the MCS was replaced with Xho I.

IFIT1F: CCGCCATGGCTACAAATGGTGATGATCATCAGG

IFIT1Xho: GCGCCTCGAGCTAAGGACCTTGTCTCACAGAGTT

The fusion protein, His-SUMO-(TEV)-IFIT1 was expressed in Rossetta in 6L LB/Amp/CM. 0.2mM IPTG was added at OD = 0.8. The expression was carried out at 18°C overnight. Purification was carried out with a routine protocol for His-tagged protein. Binding buffer contains (20mM Tris-HCl, pH8.0, 0.3M NaCl, 10mM imidazole), washing buffer contains 30mM imidazole, and protein was eluted with 300mM imidazole. To remove non-specifically bound RNA the columns were washed with 9 vol. 0.2M Na_2HPO_4/4M NaCl, pH7.5. After desalting into GF buffer (20mM Tris-HCl, pH8, 150mM NaCl, 5% Glycerol), the fusion was cleaved with TEV protease (1:100) at room temperature overnight. Gel filtration was carried out after passing through Ni-beads again and addition of 3mM DTT. The IFIT1 peak was collected and concentrated and had an A260/A280 of 0.7-0.8.

229

230 Results

231

232 **Paramyxoviruses interact in distinct ways with cells in a pre-existing IFN-induced**

233 **antiviral state.**

234

235 Despite the fact that paramyxoviruses encode IFN antagonists that inhibit IFN production

236 and signaling, their ability to block the IFN response is not absolute. Thus they form larger

237 plaques on IFN-incompetent cells than IFN-competent cells (Figure 1 and reference (19),

238 showing that during virus replication and spread some IFN is produced which slows the

239 spread of the viruses (see also Figure 3). In the experiments shown in figure 1 and below we

240 have used naïve A549, A549/Npro and A549/shIFIT1 cells; Naïve A549 cells can produce

241 and respond to IFN in response to virus infection, A549/Npro cells respond to exogenous

242 IFN but cannot produce IFN as they constitutively express Npro from bovine viral diarrhea

243 virus (BVDV) which targets IRF-3 for degradation (43). Furthermore, because IRF-3 is

244 degraded in A549/Npro cells they cannot up-regulate expression of IFIT1 in an IRF-3-

245 dependent, IFN-independent, manner in direct response to virus infection (29).

246 A549/shIFIT1 cells produce and respond to IFN but expression of endogenous IFIT1 in

247 response to IFN or viral infection is inhibited due to constitutive expression of small hairpin

248 RNA (shRNA) to IFIT1 (23).

249

250 We previously showed that IFIT1 is the major cellular protein responsible for the IFN

251 sensitivity of the rubulavirus PIV5 (23). To further investigate the ability of IFN, and the

252 role of IFIT1, to induce an antiviral state against other paramyxoviruses, we initially tested

253 the ability of PIV2, PIV3 and PIV5 to form plaques in A549, A549/Npro and A549/shIFIT1
254 cells. All three viruses induced IFN in A549 cells as the plaques developed, as observed by
255 the induction of MxA in the uninfected cells surrounding the plaque (Figure 1, panel a). As
256 previously observed (23), PIV5 formed bigger plaques on A549/shIFIT1 cells than on A549
257 cells, but the plaques were not as large as those on A549/Npro cells (Figure 1, panel b).
258 Whilst PIV2 also produced slightly larger plaques on A549/shIFIT1 than on A549 cells, the
259 plaques on A549/Npro cells were obviously bigger (note the centre of mid- to large-sized
260 PIV2 plaques has fallen out of monolayers). PIV3 produced similarly sized plaques on A549
261 and A549/shIFIT1, and slightly larger plaques on A549/Npro cells. These results also
262 support our previous conclusion that in A549 (and Hep2) cells IFIT1 is the primary ISG
263 effector to PIV5 (23) and that the rubulavirus PIV2 is also sensitive to IFIT1. However,
264 knocking down IFIT1 did not have such a marked effect on PIV2 plaque size as it did for
265 PIV5. This indicates that there are likely to be additional ISGs that play an important role in
266 IFN-mediated inhibition of PIV2. In contrast, PIV3 (Washington strain) produced similarly
267 sized plaques on A549 and A549/shIFIT1, and only slightly larger plaques on A549/Npro
268 cells; this suggests that the IFN response is capable of slowing the spread of PIV3 to some
269 degree (but not through the activity of IFIT1), but not as dramatically as it does for PIV2 or
270 PIV5. However, experiments on the JS strain of PIV3 showed it to be more sensitive to the
271 antiviral effects of IFN, but this was not because JS is sensitivity to IFIT1 (data not shown).
272
273 We next compared the synthesis of viral proteins in cells infected with PIV2, PIV3 and PIV5
274 that had, or had not, been pretreated with IFN prior to infection with PIV2, PIV3 and PIV5.
275 Cells were infected at a high moi (10-20 pfu/cell) and the relative levels of NP synthesis
276 visualized by radioactively labeling the cells for 1h with [^{35}S]-methionine at 18h p.i. (Figure

277 2). Pretreatment of A549 and A549/Npro cells with IFN in this assay reduced the expression
278 of the NP of PIV2 and PIV5 to barely detectable levels. However, IFN-pretreatment had no
279 discernable effect on the expression of the NP protein of PIV3, or on the expression of host
280 cell proteins. Strikingly, expression of NP of PIV2 and PIV5 was largely rescued in IFN-
281 pretreated A549/shIFIT1 cells, demonstrating that IFIT1 plays a major role in the inhibition
282 of PIV2 and PIV5 protein synthesis observed in A549 and A549/Npro cells pretreated with
283 IFN. Figure 2 is an exemplar of many similar experiments we have performed under
284 different conditions (time course, moi etc) that show the same result, namely that PIV2 and
285 PIV5 are inhibited by IFIT1 whilst PIV3 is not.
286
287 Having demonstrated that PIV2 and PIV5 are sensitive to IFIT1, whilst PIV3 is resistant, we
288 tested the sensitivity of other members of the Paramyxoviridae family, namely mumps virus
289 (MuV strain Enders), Sendai virus (SeV) and Canine Distemper virus (CDV). In a similar set
290 of experiments to those described in Figure 2, A549/Npro and A549/shIFIT1 cells were, or
291 were not pretreated with IFN, prior to a high multiplicity of infection with these viruses. The
292 relative levels of NP synthesis were visualized by radioactively labeling the cells for 1h with
293 [^{35}S]-methionine at 18h p.i (Figure 3, panel A). These experiments clearly demonstrated that,
294 like PIV2 and PIV5, pretreating A549 cells with IFN inhibited MuV strain Enders protein
295 synthesis, but that knocking down IFIT1 expression could largely restore MuV protein
296 synthesis. In contrast, as was observed for PIV3, although pretreatment of A549 cells with
297 IFN slightly reduced the expression of SeV and CDV protein synthesis, no increase in SeV
298 and CDV protein synthesis was observed in A549/shIFIT1 compared to A549 cells
299 pretreated with IFN. These results therefore show that MuV Enders is sensitive to IFIT1, but
300 SeV and CDV are not; the weak inhibition of SeV and CDV protein synthesis observed in

A549 and A549/shIFIT1 cells pretreated with IFN presumably being due to the action of other ISGs induced by IFN. Whilst MuV is sensitive to IFIT1, it only forms pinpoint plaques on A549/Npro cells at 5 days p.i. (data not shown), strongly suggesting that there are host cell restrictions other than innate intracellular defence mechanisms on MuV replication in A549 cells (44).

Since in these experiments we used the attenuated Enders strains of MuV to test whether attenuation may be linked to sensitivity to IFIT1, we tested a wild type isolate of MuV-London-1 (Lo-1) for its sensitivity. At the same time we also tested the sensitivity of another strain of PIV5, termed CPI+ (Figure 3, panel B). MuV-Lo was as sensitive as MuV Enders, demonstrating that attenuation was not linked to differences in their relative sensitivity to IFIT1. Similarly PIV5 CPI+ was also sensitive to inhibition by IFIT1.

The IFIT1-sensitivity of PIV5 is not rescued by co-infection with an IFIT1-resistant virus.

From these results it was clear that the replication of the non-rubulaviruses PIV3, SeV and CDV are not inhibited by IFIT1. To investigate whether PIV5 replication could be rescued by co-infections with an IFIT1-resistant virus, mixed infections between PIV3 and PIV5 were undertaken. To avoid any possible synergistic effects between PIV3 and PIV5 in dismantling an IFN-induced antiviral state, the CPI- strain of PIV5 was used in these experiments as, due to mutations in its V protein, it does not block IFN signaling (45). A549 or A549/shIFIT1 cells were or were not pretreated with IFN for 8h prior to high multiplicity (10 - 20 p.f.u./cell) infection with PIV5, PIV3, or a mixture of both viruses (Figure 4, panel a). The expression of the NP protein of PIV3 was resistant to IFN in both A549 and in

A549/shIFIT1 cells when they were infected with PIV3 alone and when co-infected with PIV5. In contrast, whilst the expression of PIV5 NP was resistant to IFN in A549/shIFIT1 cells, its expression was inhibited in A549 cells, even when the cells were co-infected with PIV3. Immunofluorescence was undertaken to ensure that in these experiments there was no exclusion of one virus by the other (Figure 4, panels b and c). These results confirmed that co-infection of PIV3 with PIV5 does not rescue the sensitivity of PIV5 to IFIT1 and strongly suggests that PIV3 does not specifically inhibit the antiviral activity of IFIT1, and that the inhibition of PIV5 NP expression is regulated by cis-acting elements.

Differential inhibition of translation of mRNAs of different paramyxoviruses by purified IFIT1

The data above show that the IFN-sensitivity of rubulaviruses is at least in part due to the actions of IFIT1. Since this cellular protein has been shown to inhibit translation in a template-specific manner we developed an *in vitro* translation system to study the ability of human IFIT1 to selectively inhibit the translation of rubulavirus mRNAs. The gene encoding human IFIT1 was cloned as an SUMO-fusion protein expressed in E.coli and the recombinant protein purified (Figure 5, panel a). To determine whether the recombinant IFIT1 was able to selectively inhibit PIV5 mRNAs, *in vitro* translation of mRNA isolated from mock and PIV5-infected cells was carried out in the presence and absence of different concentrations of IFIT1 (Figure 5, panels b, c and d). In the absence of IFIT1, expression of the NP protein (and to a lesser extent the M protein) of PIV5 could clearly be visualized in the background of *in vitro* translated cellular proteins (Figure 5 panels c and d). Increasing concentrations of IFIT1 had no obvious effect on the efficiency of translation of host cell

proteins, but in striking contrast, purified IFIT1 selectively inhibited the translation of the NP and M proteins of PIV5 in a concentration-dependent manner.

Having established that the sensitivity of *in vitro* translation of PIV5 mRNA to inhibition by purified IFIT1 correlated with the biological sensitivity of PIV5 to IFIT1, we next tested the ability of IFIT1 to inhibit the translation of mRNA isolated from cells infected with other paramyxoviruses (Figure 6). These results clearly demonstrated that translation of (NP) mRNAs from PIV2- and from MuV-infected cells was inhibited by IFIT1. In contrast, there was no obvious reduction in the amount of PIV3 NP synthesized when increasing amounts of IFIT1 was added to the *in vitro* translation reactions. Although there was a slight apparent reduction in the amount of SeV and CDV NP synthesis in the samples in which IFIT1 was added, there was no increase the inhibition observed by increasing the amount of IFIT1 added to the *in vitro* translation reactions, strongly suggesting that the translation of SeV and CDV mRNAs are also resistant to inhibition by IFIT1.

Lack of 2'-O methylation of the cap structure of MuV and PIV5 mRNAs is partially responsible for their sensitivity to inhibition by IFIT1

Previous studies have shown that the absence of cap 1 on mRNAs renders them sensitive to inhibition to IFIT1. To investigate whether this was the case for rubulavirus mRNAs we developed an *in vitro* assay in which purified human mRNA-modifying enzymes were used to progressively cap and add different methyl groups to the 5'ends of mRNAs. Purified human RNA guanylyltransferase and 5'-phosphatase (RNGTT), RNA guanine-7 methyltransferase (RNMT) and cap methyltransferase 1 (CMTR1) were used in these assays.

RNGTT adds a 5’ guanosine to RNAs with 5’-ppp, whilst RNMT adds a methyl group to the ^{7}G of the guanine ring, generating ($m^{7}G$) cap 0. CMTR1 adds a methyl group to the 2’ OH position of the adjacent ribose, generating cap 1. To demonstrate the functionality of this system, we first tested the *in vitro* translation of luciferase mRNA with a 5’-triphosphate group. This RNA was efficiently translated in a cap-independent manner and was only weakly inhibited by IFIT1 (Figure 7a, compare lanes 1 and 2). When the luciferase mRNA was capped with the addition of 5’-guanosine by RNGTT (generating Gppp-mRNA) there was a slight decrease in the amount of luciferase made (Figure 7a, compare lanes 1 and 3). This may have been due to RNGTT destabilizing or blocking the translation of Gppp-mRNAs in the absence of ^{7}N methylation. However strikingly, translation of this mRNA was completely inhibited by IFIT1(Figure 7a lane 4) despite this cap structure lacking N-7 methylation. As expected, the addition of a methyl group to the N-7 position of the guanine ring (generating $m^{7}Gpppm^{2}N$) by RNMT increased the efficiency of translation, but m^{7}Gppp-luciferase remained completely sensitive to inhibition by IFIT1 (Figure 7a lanes 5 and 6). Addition of a methyl group to the 2’ OH group of the adjacent ribose (generating cap 1) by CMTR1 did not affect the efficiency by which the mRNA is translated but it did clearly reduce the sensitivity of the mRNA to inhibition by IFIT1(Figure 7A compare lanes 7 and 8). However, it should be noted that in these experiments, for reasons that are unclear, we were unable to completely restore full translation of the luciferase mRNA in the presence of IFIT1 by increasing the amount of CMTR1 or length of incubation of the mRNA with the enzyme (data not shown).

To investigate how similar modifications to the cap of rubulavirus mRNAs influenced their inhibition by IFIT1, we initially used MuV mRNA in a parallel set of experiments. These

results showed that treatment of the MuV mRNA with RNGTT and RNMT did not increase the efficiency of *in vitro* translation of MuV NP mRNA or its sensitivity to inhibition by IFIT1 (Figure 7b lanes 1 to 6), consistent with the viral polymerase adding m^7Gppp-cap at (cap 0) to the 5' end of viral mRNAs. However, surprisingly, since rubulavirus polymerases have conserved 2'-O MTase domains, addition of a methyl group to the 2'OH group of the adjacent ribose (cap 1) by CMTR1 clearly reduced the sensitivity of the NP mRNA to inhibition by IFIT1 (Figure 7b lanes 7 and 8). As expected, the IFIT1 sensitivity was dependent on the addition of S-adenosyl methionine (SAM) to the reaction mixture (Figure 7c). Similarly, following 2'O methylation of PIV5 mRNA, *in vitro* translation of PIV5 NP became completely resistant to inhibition by IFIT1 (Figure 7d). Strikingly, in contrast to NP, the translation of PIV5 M mRNA remained completely sensitive to inhibition by IFIT1 even after 2'O methylation of PIV5 mRNA by CMTR1 (Figures 7d and 7e); the basis for this is currently unknown but we are investigating it further.

Discussion

Over the past decade or so it has become clear that the ways in which paramxoviruses circumvent innate immune responses, including the IFN response, and differences in the multifunctional nature of their IFN antagonists are likely to influence the types of disease they cause. For example, the viral IFN antagonists within the rubulavirus genus, namely the V proteins, as well as interacting with common targets such as MDA 5 and LGP2 also have unique properties. The V protein of PIV5 targets STAT1 for degradation, PIV2 targets STAT2 and MuV targets both STAT1 and STAT3. Within the respirovirus and morbillivirus genera it is a combination of the V and C proteins that counteract innate responses by

421 different molecular mechanisms and strikingly, although PIV3 encodes a C protein, it does
422 not encode a functional V protein. Despite encoding these powerful IFN antagonists, IFN is
423 produced during virus spread both in tissue culture cells and *in vivo*, and thus undoubtedly
424 paramyxoviruses will, during the course of an infection, infect cells in a pre-existing IFN-
425 induced antiviral state. Here we show that different paramyxoviruses interact in distinct
426 ways with cells in a pre-existing IFN-induced antiviral state, and suggest that this may
427 influence the types of diseases caused. Strikingly, in contrast to the sensitivity of
428 rubulaviruses to IFIT1, the other paramyxoviruses we tested were resistant, strongly
429 suggesting that this might be a distinguishing feature of rubulaviruses, although before this
430 can be firmly concluded the sensitivity of more species of paramyxoviruses to IFIT1 needs
431 to be tested. Even within the rubulavirus genus it appears that there may be differences in
432 how members interact with cells in IFN-induced antiviral state. In A549 cells IFIT1 is
433 primarily responsible for the IFN-induced antiviral state induced to counter PIV5. However,
434 although PIV2 is sensitive to IFIT1, there appear to be other ISGs that have strong anti-PIV2
435 activity. This conclusion comes from the observation that whilst there is a slight increase in
436 the size of PIV2 plaques on A549/shIFIT1 cells compared to A549 cells, it is not as obvious
437 as that observed for PIV5. Furthermore, whilst plaques for PIV5 were smaller on
438 A549/shIFIT1 cells than on A549/Npro cells, this difference was not as marked as that
439 observed for PIV2. MuV Enders strain is also sensitive to IFIT1, but there are clearly other
440 major constraints on the growth of MuV Enders in human cells as the virus grows extremely
441 poorly in IFN-incompetent human cells but replicates to high titres in Vero cells (44).
442
443 It is striking that only rubulaviruses are sensitive to the antiviral activity of human IFIT1.
444 Our data indicate that the inhibition of rubulavirus mRNAs was inhibited by IFIT1 in a cis-

445 linked manner, implying that the restriction is associated with some feature of the mRNA
446 sequence or structure. Since IFIT1 can selectively inhibit the translation of mRNAs that are
447 incorrectly capped or not methylated at the 2' OH group of the first ribose, i.e. cap 1, (24, 46,
448 47), it was likely that rubulaviruses have a structural motif in their cap, not present or hidden
449 in the mRNA of other paramyxoviruses, that is recognized by IFIT1. To investigate this
450 further we used purified human enzymes to modify the cap of mRNAs. As a control for the
451 activity of the enzymes we used an uncapped 5'-ppp mRNA that encodes luciferase. The 5'-
452 ppp luciferase mRNA translated in a cap-independent manner *in vitro* using rabbit
453 reticulocyte lysate and this translation was only weakly inhibited by purified IFIT1. Whilst
454 addition of a 5' guanosine nucleoside cap slightly decreased the amount of luciferase
455 synthesized, probably because the enzyme RNGTT destabilizes the mRNA, addition of the
456 (unmethylated) guanosine nucleoside to the 5' end of the mRNA significantly increased the
457 sensitivity of the mRNA to inhibition by IFIT1. Furthermore, although methylation of the
458 guanine ring at position N7 (m7GpppNp-RNA) by RNMT increased the efficiency of
459 translation of luciferase mRNA it did not appear to affect the sensitivity of inhibition by
460 IFIT1.These results are therefore consistent with the observation that human IFIT1 binds
461 with low affinity to 5'-ppp RNA, but more avidly to cap 0 RNA lacking 2' O methylation.
462 Methylation at position N7 of the guanine ring has also been reported to increases the
463 affinity of binding of IFIT1 (24), however the observation here that Gppp-luciferase is
464 inhibited as efficiently as m7Gppp-luciferase suggests that the methyl group does not play a
465 central role in the inhibition of mRNAs by IFIT1. In contrast, 2' O-methylation of the first
466 ribose by CMTR1 to generate cap 1 partially prevented IFIT1 from inhibiting the translation
467 of the cap 0-modified mRNA. However, even by increasing the amount of CMTR1 and the
468 incubation time we were unable to complete restore full translational activity of the

469 luciferase mRNA. The reasons for this are unclear but it suggests that other structural
470 features, for example methylation of the penultimate ribose to generate cap 2, or sequences
471 at the 5' end of mRNAs, may also influence inhibition by IFIT1, as has been suggested by
472 Daugherty et al (30).
473
474 mRNAs isolated from PIV3, SeV and CDV infected cells were not inhibited by IFIT1 and
475 neither was the replication of these viruses (Figures 2 and 3). In contrast, 2' O-methylation
476 of the terminal ribose by CMTR1 of MuV mRNAs partially alleviated inhibition of the NP
477 mRNA by IFIT1. With regards PIV5, our previous studies suggested that PIV5 mRNAs
478 were 2' O-methylated (23). Furthermore, we never observed complete IFIT1-inhibition of
479 PIV5 NP synthesis *in vitro,* suggesting that at least a proportion of the PIV5 NP mRNA was
480 correctly capped. However, the fact that treating PIV5 mRNAs with CMTR1 rescued NP
481 synthesis in the presence of IFIT1 suggests that a significant proportion of PIV5 mRNAs
482 was also not fully methylated. It is also of potential significance that the M mRNA of PIV5
483 appears to be more sensitive than NP mRNA to inhibition by IFIT1 and furthermore
484 translation inhibition of PIV5 M mRNA was not rescued by treatment with CMTR1. The
485 reasons for differences in the relative sensitivity of the NP and M mRNAs clearly warrants
486 further investigation, but may be due to the fact that the viral methyltransferase differentially
487 methylates the viral mRNAs (as has been shown for VSV (48)), that CMTR1 does not
488 recognize the UTR of the PIV5 M mRNA, or that inhibition by IFIT1 is influenced by
489 additional structural features present on PIV5 M mRNA but not NP mRNA. Regarding this
490 latter point, it is of note that the first three nucleotides of the UTRs of NP and M differ.
491 Furthermore the 4 to 5 nucleotides downstream of cap 0 are thought to be bound by IFIT1
492 and may thus modulate IFIT1-RNA interactions (49) and some secondary RNA structures,

493 e.g. those found at the 5’ end of some alphaviruses, can prevent IFIT1 binding to RNA
494 independent of the cap-methylation status (50).
495
496 Most viruses successfully avoid inhibition by IFIT1 by encoding their own 2’-O MTase ,
497 cap-snatching appropriately capped and 2’-O methylated structures from cellular mRNAs or
498 having cap-independent translation with covalently linked viral protein, VPg or a 5’ RNA
499 secondary structure that block the activity of IFIT1 (reviewed in (26)). Indeed, work on
500 virus restriction by IFIT1 has primarily involved the investigation of viruses in which the 2’-
501 O-MTases have been mutated such that their mRNAs do not have a cap 1 structure (25, 51-
502 54). Nevertheless our results show that the viral polymerase of rubulaviruses, unlike other
503 paramyxoviruses, does not fully protect the viral mRNAs from inhibition by human IFIT1.
504 In this regard, it is of interest to note that, although rubulaviruses have the conserved
505 methyltransferase domain in their polymerase, they all have an alanine instead of the first
506 glycine in a GxGxG motif present in the methyltransferase domain of other paramyxoviruses
507 and mononegavirales that has been shown to affect the efficiency of cap methylation (55).
508
509 Most viruses, including other mononegavirales (56), appear to be naturally resistant to
510 inhibition by IFIT1. It is therefore intriguing that rubulaviruses have not evolved
511 mechanisms to ensure that their mRNAs are correctly capped and methylated, or have the
512 appropriate UTRs, to be resistant to IFIT1. It is tempting to speculate that there is some
513 unknown biological advantage to being sensitive to IFIT1. For example, it may help some
514 rubulaviruses (and perhaps hepatitis C virus (57) which is also sensitive to IFIT1) to
515 establish prolonged or persistent infections. Thus, following infection of cells in an IFN-
516 induced antiviral state, IFIT1 restricts PIV5 replication. Under such conditions, virus

517 genomes are located in cytoplasmic foci where, as we have previously suggested, they may
518 remain hidden from intracellular and adaptive immune responses. Furthermore, if viral
519 mRNA is produced in cells in an IFN-induced antiviral state then viral protein synthesis will
520 largely be inhibited by IFIT1, thus reducing the amount of protein that may be processed and
521 presented to CTLs. Eventually, in such cells however, enough of the virus IFN antagonist,
522 the V protein, will be produced, or brought in by infecting virus particles, to target STAT1
523 for proteasome-mediated degradation, and the cells will no longer be able to maintain their
524 antiviral state, thus facilitating virus replication (58). Whether such a scenario occurs *in*
525 *vivo*, these, and other considerations, emphasize that to fully understand the molecular
526 pathogenesis of viruses, it will be necessary to understand the subtleties of how viruses
527 interact with the IFN system and other host cell defence mechanisms.
528

Acknowledgements

This work was supported by The Welcome Trust (101788/Z/13/Z, 101792/Z/13/Z) and Medical research council grant (G1100110/1, MR/K024213/1). The University of St Andrews is a charity registered in Scotland (No.SC013532).

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Figure legends

Figure 1

Panel a). A549 cells produce and respond to IFN during development of PIV2, PIV3 and PIV5 plaques. Panel a). A549 cells grown on coverslips in 24 well microtitre were infected with PIV2, PIV3 or PIV5 at a multiplicity that resulted in 2 – 10 plaques per well. At 3 days p.i. the cells were fixed and stained with antibodies to the NP proteins of the respective viruses (green) and anti-MxA antibody, which is an ISG that is up-regulated in response to IFNα/β (red). The images show cross sections through plaques with the center of the plaque at the left hand side of the image. Panel b). Relative plaque size of PIV2 (Colindale), PIV3 (Washington strain) and PIV5 (W3) on A549, A549/shIFIT1 and A549/NPro cells. Infected monolayers of cells in 6-well dishes were fixed at 4 days (PIV3) or 5 days (PIV2 and PIV5) p.i. and virus plaques visualized by immunostaining the monolayers with antibodies to the respective NP and/or P proteins. The numbers at the bottom left in each panel give the average plaque size in mm, together with their standard deviation.

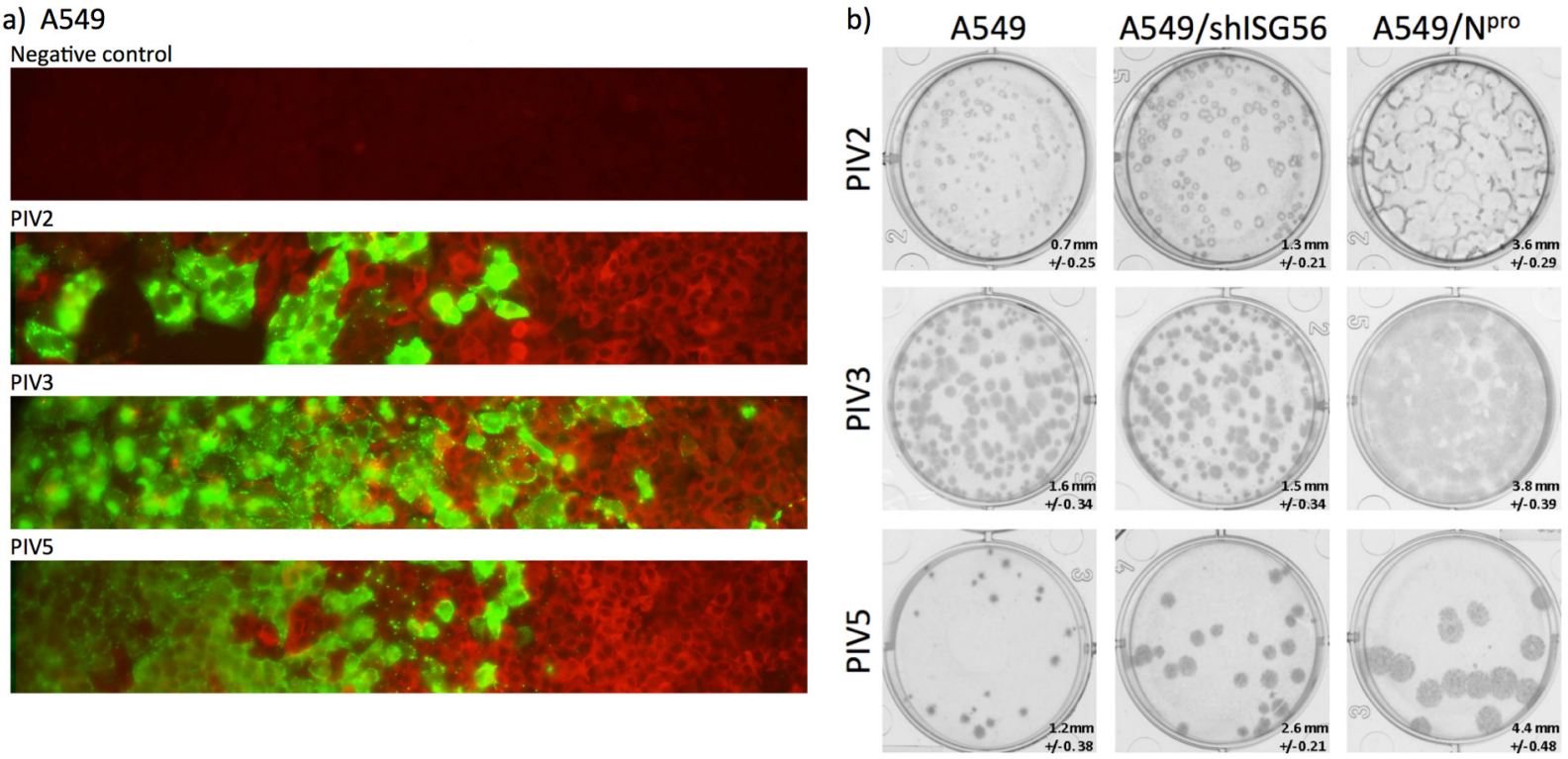
Figure 2

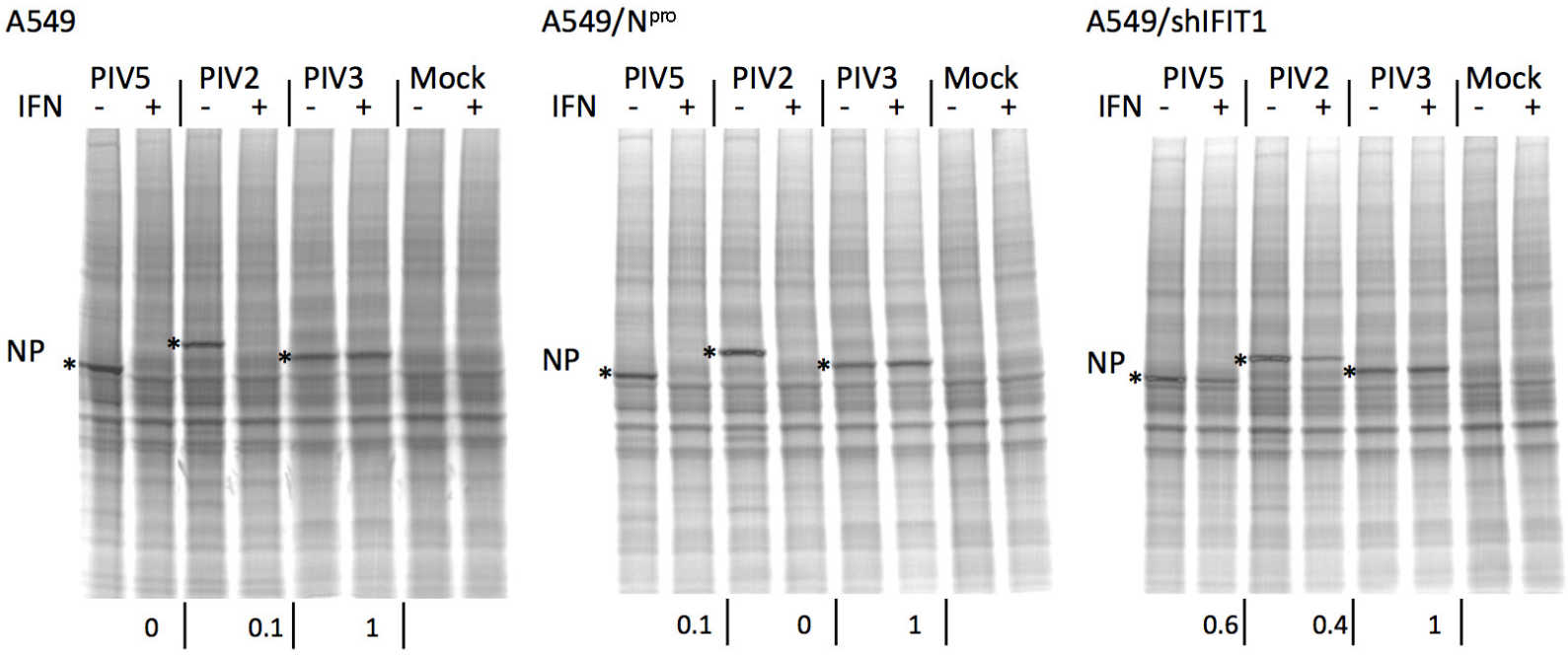
729 IFIT1 inhibits PIV5 (W3) and PIV2 (Colindale) protein synthesis but not that of PIV3
730 (Washington). A549, A549/ NPro cells and A549/shIFIT1 cells, grown in 25-cm^2 flasks
731 were, or were not, treated with IFN 8h prior to infection at a high moi with PIV2, PIV3
732 (Washington strain) or PIV5. At 18h p.i. cells were metabolically labeled with [^{35}S]-
733 methionine for 1h. Total-cell extracts were separated by electrophoresis through a 4 -12%
734 PAG and labeled proteins visualized using a phosphoimager. The positions of the NP
735 polypeptides are indicated by asterisks. The figures at the bottom indicate the fraction of NP
736 made in cells pretreated with IFN compared to untreated cells as estimated by densitometry
737 scans.
738
739
740 Figure 3
741 IFIT1 inhibits MuV (Enders and wild type (Lo-1) strains), PIV5 (strain CPI+) protein
742 synthesis but not that of SeV or CDV. A549/ N^{Pro} cells and A549/shIFIT1 cells, grown in 25-
743 cm^2 flasks, were or were not treated with IFN 8h prior to infection at high multiplicity with
744 MuV Enders, MuV Lo-1 (wt), SeV, CDV or PIV5 (CPI+). At 18h p.i. cells were
745 metabolically labeled with [^{35}S]-methionine for 1h. Total-cell extracts were separated by
746 electrophoresis through a 4 -12% PAG and labeled proteins visualized using a
747 phosphoimager. The figures at the bottom indicate the fraction of NP made in cells
748 pretreated with IFN compared to untreated cells.
749
750 Figure 4
751 PIV3 (Washington) does not inhibit the antiviral activity of IFIT1. A549 and A549/shIFIT1
752 cells, grown in either 25-cm^2 flasks (panel a) or on coverslips (panels b and c), were or were

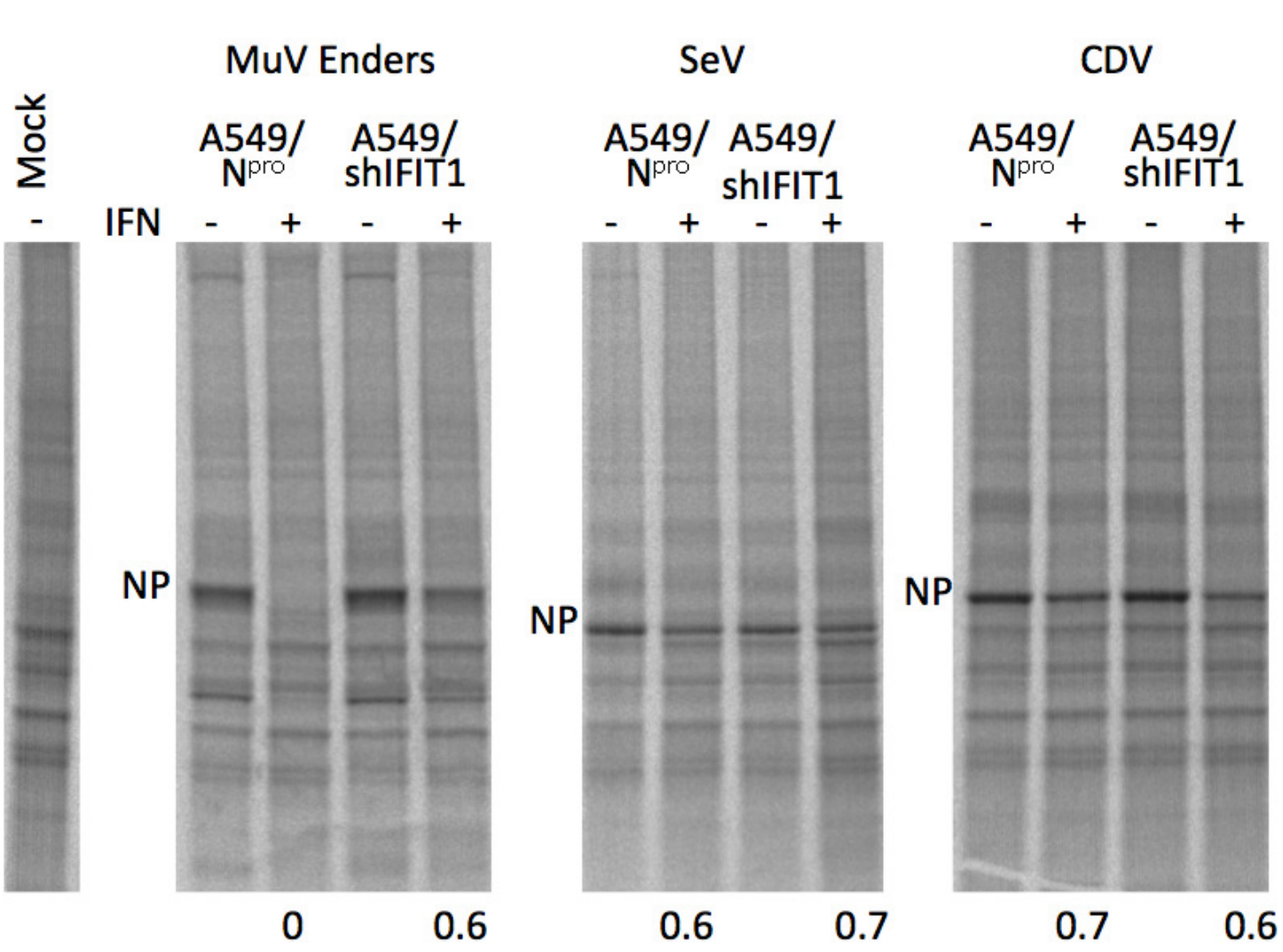
753 not treated with IFN 8h prior to infection at high multiplicity with PIV3, PIV5 (strain CPI-)
754 or a mixture of both viruses. Panel a): At 18h p.i. the cells were metabolically labeled with
755 [^{35}S]-methionine for 1h. Total cell extracts were separated by electrophoresis through a 4 -
756 12% PAG and labeled proteins visualized using a phosphoimager. The positions of the NP
757 protein are indicated by asterisks. Panel b and c): At 18h p.i. cells grown on coverslips were
758 fixed and immunostained with antibodies specific for the NP and/or P proteins of the
759 respective viruses.
760
761 Figure 5
762 Purified IFIT1 directly inhibits *in vitro* translation of PIV5 mRNA. Panel a) Coomassie
763 Brilliant Blue-stained PAG of purified IFIT1, molecular weight markers are shown in the left
764 hand lane. Panels b) and c). RNA isolated from mock- or PIV5-infected cells was *in vitro*
765 translated in the presence of [^{35}S]-methionine for 90 min in the presence or absence of
766 increasing concentrations of purified IFIT1 (0.1 and 1.0 μg per reaction mixture).
767 Polypeptides were separated by electrophoresis through a 4 -12% PAG. The total protein
768 content present in the *in vitro* translation mixes were visualized by staining the gel with
769 Coomassie Brilliant Blue (panel b) and the radioactively labeled proteins (panel c) visualized
770 using a phosphoimager. Panel d shows the densitometry traces of lanes 1, 3, 4 and 6 of panel
771 c. The position of PIV5 NP and M proteins are indicated; asterisks mark two prominent host
772 cell polypeptides. The figures at the bottom of the gel indicate the fraction of either the host
773 cell proteins or NP proteins made in the *in vitro* translation mixes in the presence of purified
774 IFIT1 compared to in the absence of IFIT1.
775
776 Figure 6

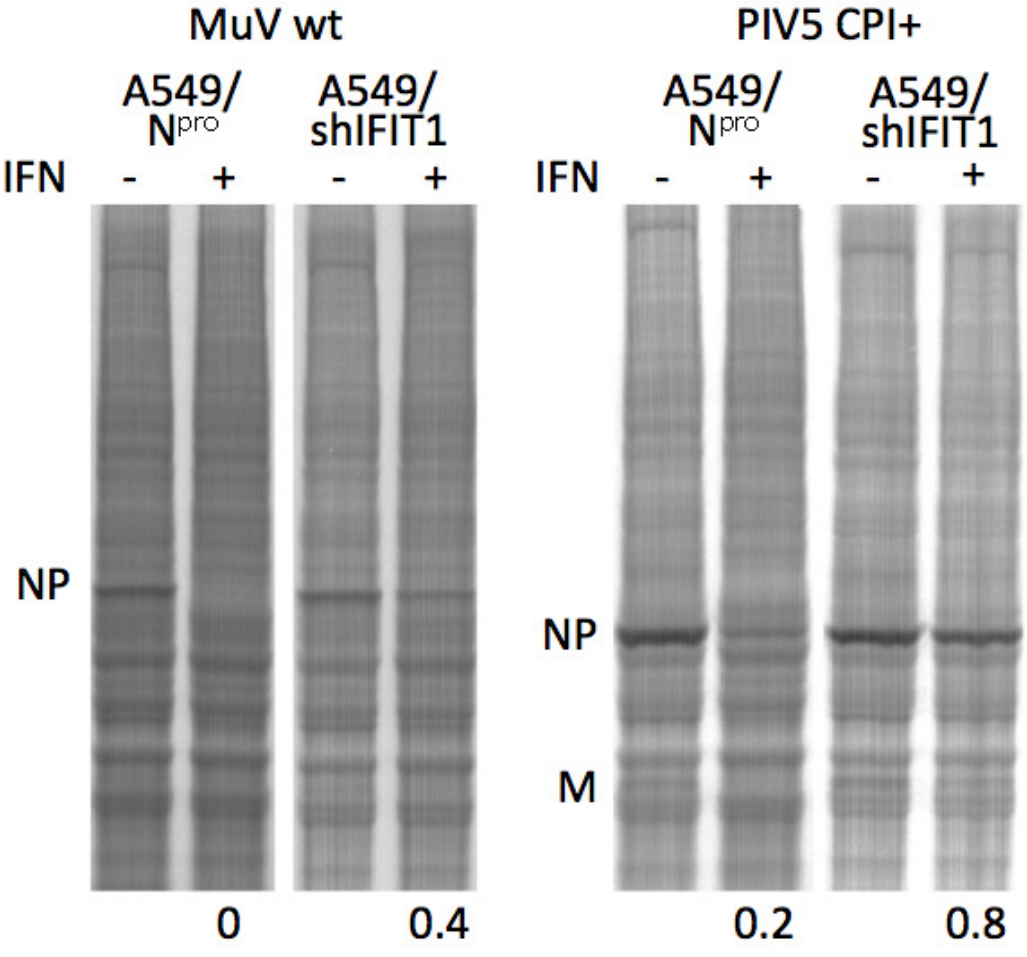
777 Purified IFIT1 inhibits the *in vitro* translation of NP mRNA isolated from PIV2-, and MuV-
778 infected cells but not mRNA from mock-infected cells or NP mRNA isolated from PIV3-
779 (Washington strain), SeV- or CDV- infected cells. RNA isolated from mock or infected cells
780 was *in vitro* translated in the presence of [^{35}S]-methionine for 90 min in the presence or
781 absence of increasing concentrations of purified IFIT1 (0.1 and 1.0μg per reaction mixture).
782 Polypeptides were separated by electrophoresis through a 4 -12% PAG and labeled proteins
783 visualized using a phosphoimager. The positions of the NP proteins are indicated. The
784 figures at the bottom of the gel indicate the fraction of either the host cell proteins or NP
785 proteins made in the *in vitro* translation mixes in the presence of purified IFIT1 compared to
786 in the absence of IFIT1.
787
788 Figure 7
789 Lack of 2'-O methylation of the cap 1 structure of MuV and PIV5 mRNAs is at least
790 partially responsible for their sensitivity to inhibition by IFIT1. Panel a): uncapped 5'-ppp
791 mRNA encoding luciferase synthesized by T7 polymerase (provided as a control in the
792 Promega *in vitro* translation kit) was translated *in vitro* in a rabbit reticulocyte lysate in the
793 absence or presence of purified IFIT1 (lanes 1 and 2). RNGTT was used to add a 5' guanine
794 cap (lanes 3 and 4), then RNMT to methylate the cap at the N7 position (lanes 5 and 6),
795 generating cap 0, and CMTR1 to methylate the adjacent ribose on the 2' OH position,
796 generating (cap 1). The modified mRNAs were then *in vitro* translated in the absence (lanes
797 3, 5 and 7) or presence of IFIT1 (lanes 4, 6 and 8). Panel b): mRNA isolated from MuV-
798 infected cells was treated in parallel under the same conditions as described for panel (a).
799 Panel c and d): mRNA isolated from either MuV- (Enders) or PIV5- (W3) infected cells was
800 *in vitro* translated prior to (lanes 1 and 2) or following modification by CMTR1 in the

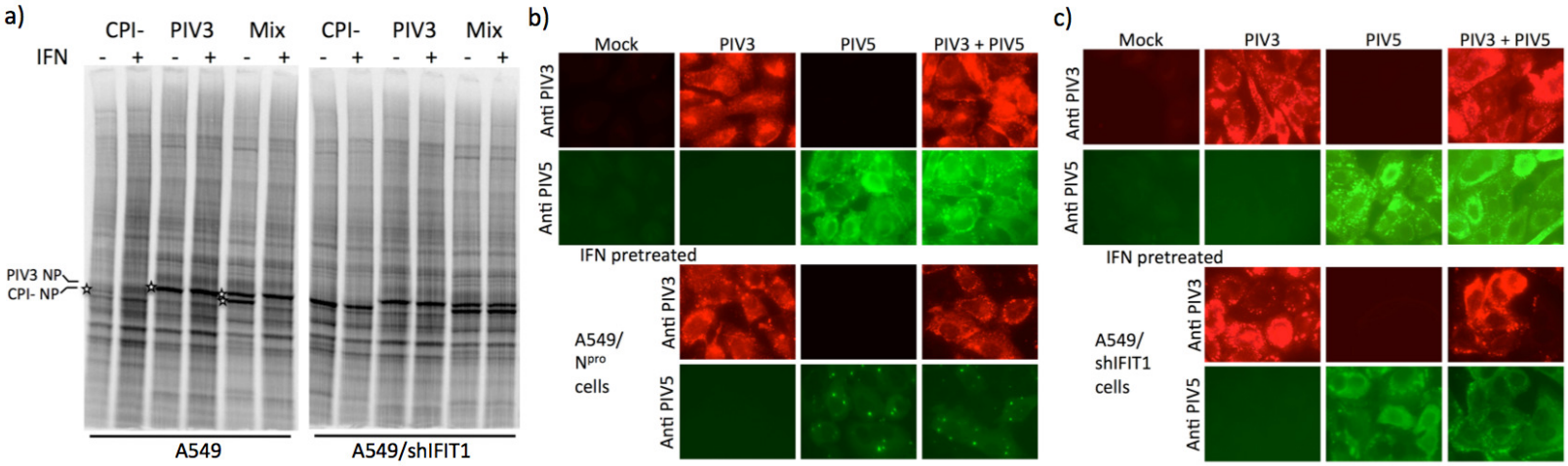
801 presence (lanes 3 and 4) or absence (lanes 5 and 6) of SAM. The mRNA was also translated
802 in the absence (lanes 1, 3 and 5) or presence of purified IFIT1 (lanes 2, 4 and 6). Panel e)
803 shows the densitometry traces of lanes 1, 2, 3 and 4 of panel d). The figures at the bottom of
804 the gel indicate the fraction of either the host cell proteins or NP proteins made in the *in vitro*
805 translation mixes in the presence of purified IFIT1 compared to in the absence of IFIT1.

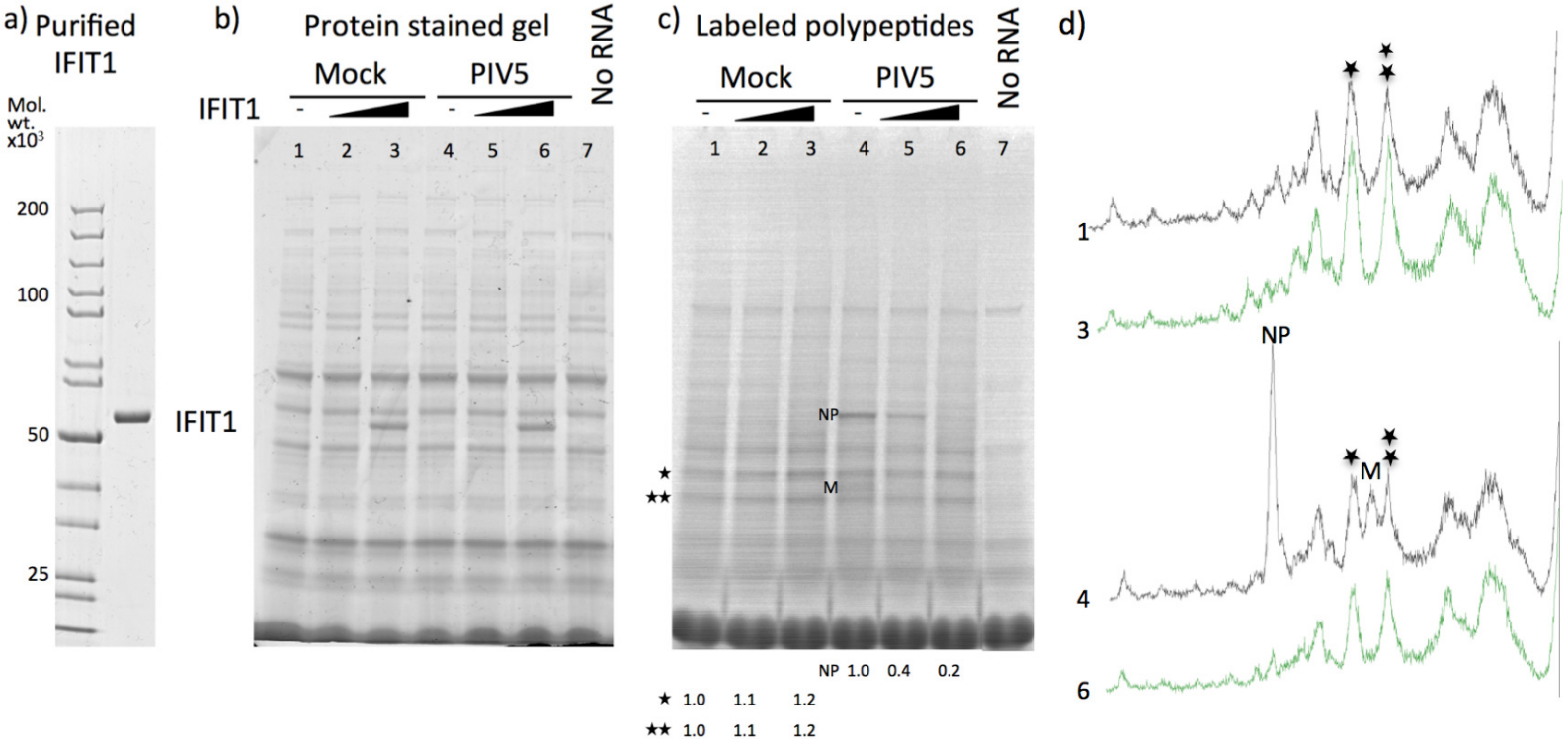

a) A549
Negative control
PIV2
PIV3
PIV5
b)
A549
A549/shISG56
A549/Npro
PIV2
PIV3
PIV5
0.7 mm +/-0.25
1.3 mm +/-0.21
3.6 mm +/-0.29
1.6 mm +/-0.34
1.5 mm +/-0.34
3.8 mm +/-0.39
1.2mm +/-0.38
2.6 mm +/-0.21
4.4 mm +/-0.48

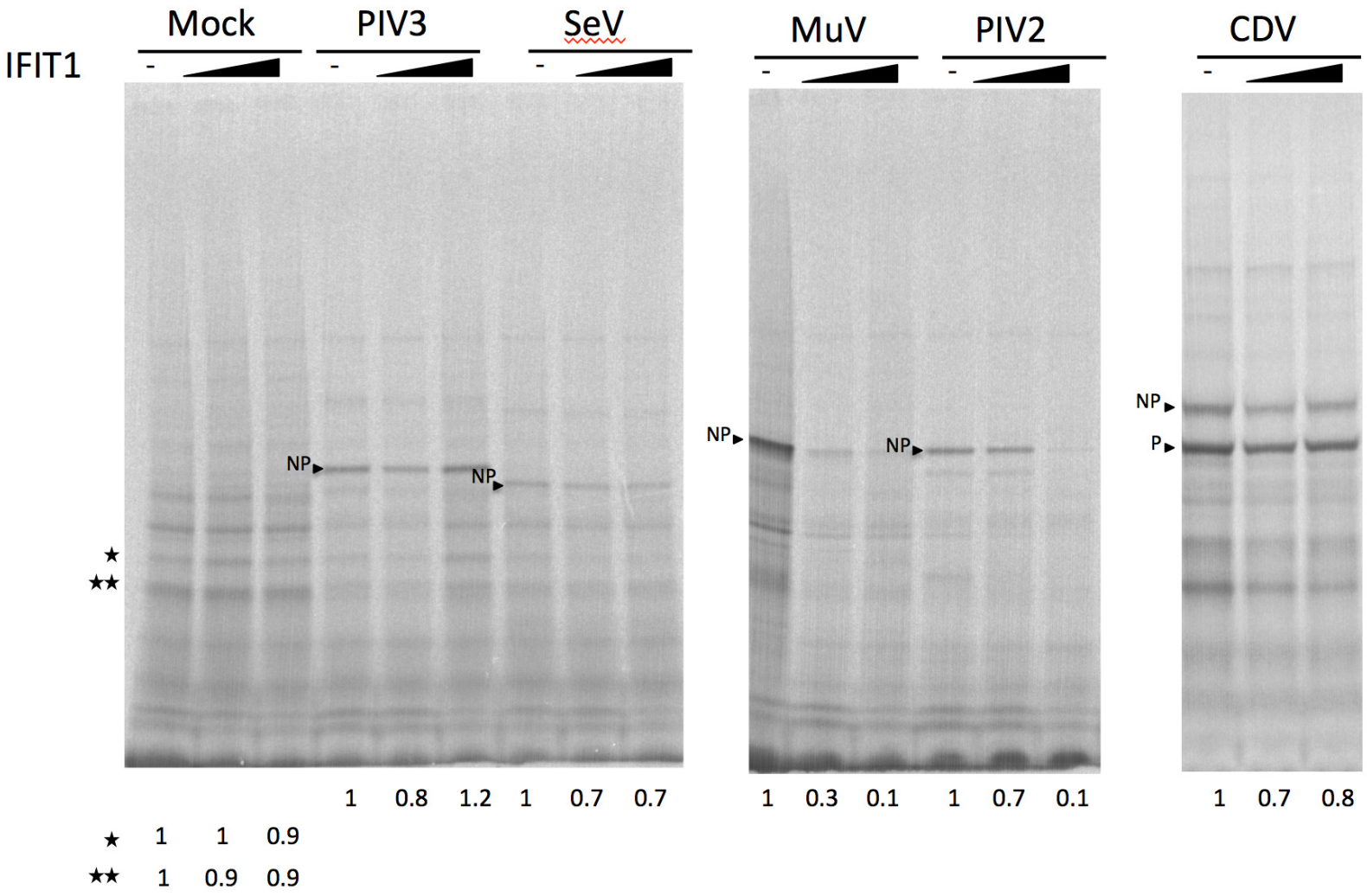

A549
PIV5
PIV2
PIV3
Mock
IFN
- + - + - + - +
NP
*
0
0.1
1
A549/N^pro
PIV5
PIV2
PIV3
Mock
IFN
- + - + - + - +
NP
*
0.1
0
1
A549/shIFIT1
PIV5
PIV2
PIV3
Mock
IFN
- + - + - + - +
NP
*
0.6
0.4
1

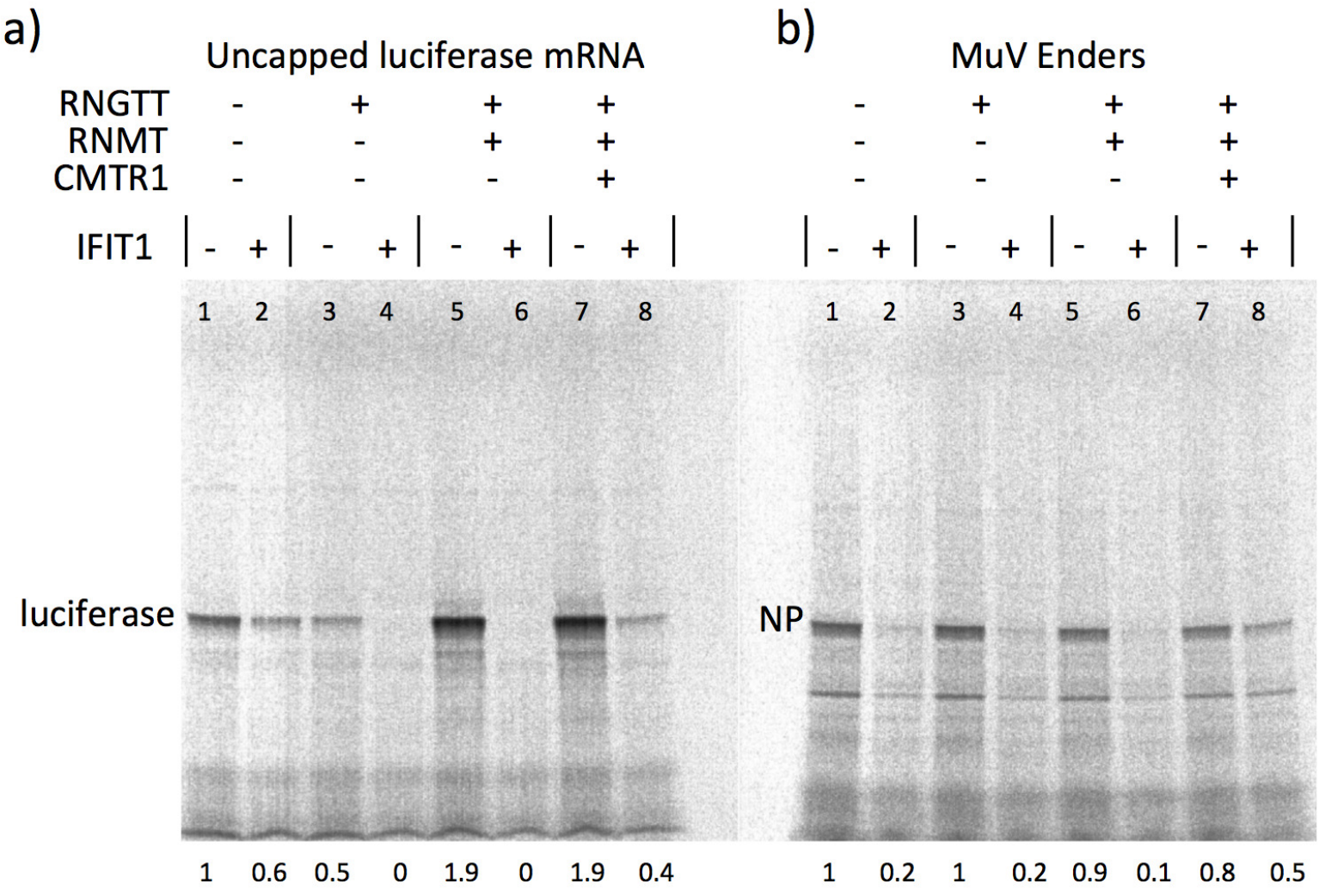

MuV Enders
SeV
CDV
Mock
A549/ Npro
A549/ shIFIT1
IFN
-
+
NP
0
0.6
0.6
0.7
0.7
0.6

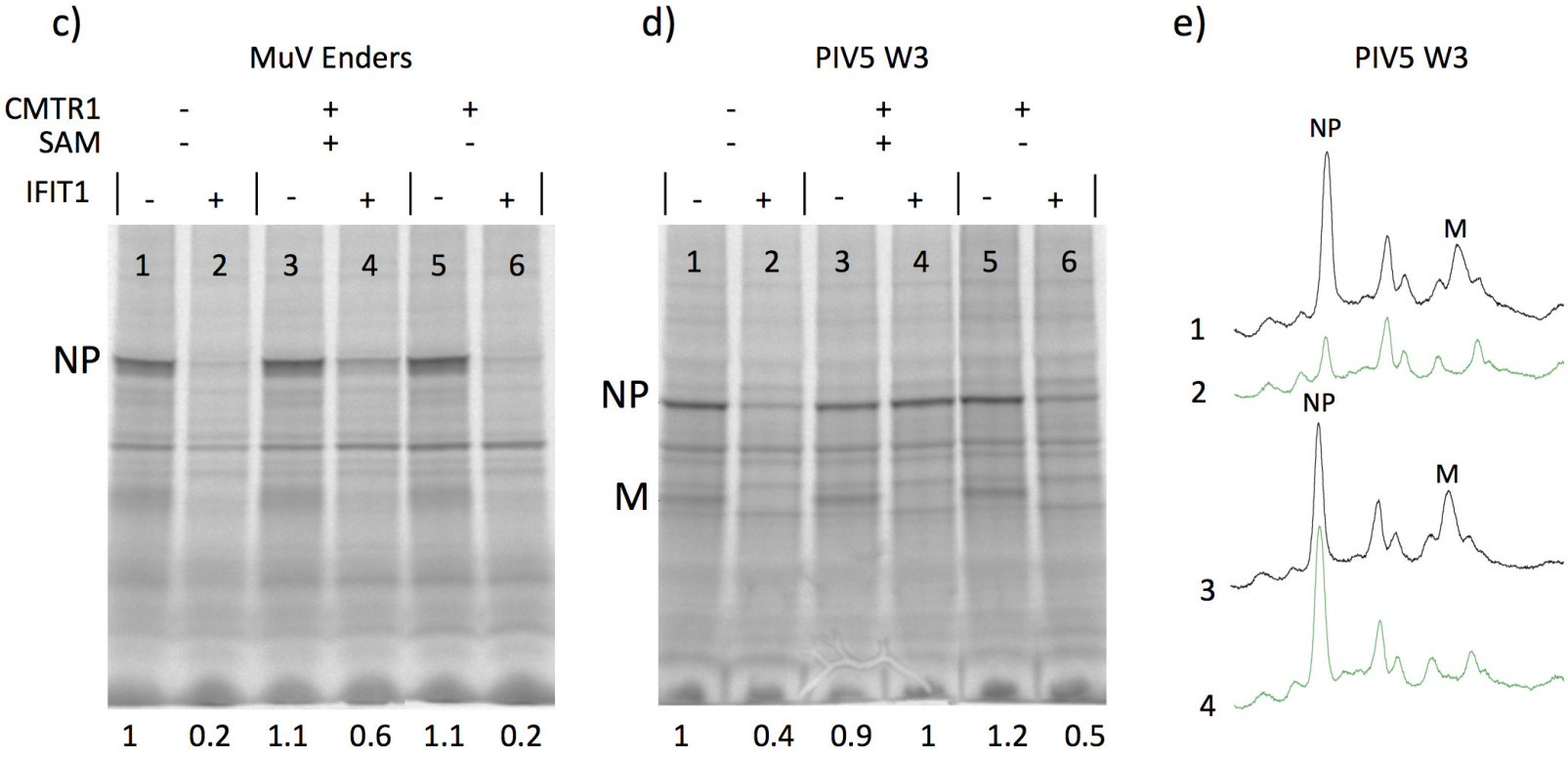

MuV wt
PIV5 CPI+
A549/ Npro
A549/ shIFIT1
IFN
NP
M
0
0.4
0.2
0.8


a)
CPI-
PIV3
Mix
CPI-
PIV3
Mix
IFN
- + - + - + - + - + - +
PIV3 NP
CPI- NP
A549
A549/shIFIT1
b)
Mock
PIV3
PIV5
PIV3 + PIV5
Anti PIV3
Anti PIV5
IFN pretreated
A549/
N^pro
cells
Anti PIV3
Anti PIV5
c)
Mock
PIV3
PIV5
PIV3 + PIV5
Anti PIV3
Anti PIV5
IFN pretreated
A549/
shIFIT1
cells
Anti PIV3
Anti PIV5


a) Purified IFIT1
Mol. wt. x10^3
200
100
50
25
b)
Protein stained gel
Mock
PIV5
No RNA
IFIT1
1 2 3 4 5 6 7
IFIT1
c) Labeled polypeptides
Mock
PIV5
No RNA
1 2 3 4 5 6 7
NP
M
★
★★
NP 1.0 0.4 0.2
★ 1.0 1.1 1.2
★★ 1.0 1.1 1.2
d)
1
3
NP
M
4
6


IFIT1
Mock
PIV3
SeV
MuV
PIV2
CDV
-
NP
P
★
★★
1 0.8 1.2 1 0.7 0.7
1 0.3 0.1 1 0.7 0.1
1 0.7 0.8
★ 1 1 0.9
★★ 1 0.9 0.9


a)
Uncapped luciferase mRNA
RNGTT - + + +
RNMT - - + +
CMTR1 - - - +
IFIT1 - + - + - + - +
1 2 3 4 5 6 7 8
luciferase
1 0.6 0.5 0 1.9 0 1.9 0.4
b)
MuV Enders
- + + +
- - + +
- - - +
- + - + - + - +
1 2 3 4 5 6 7 8
NP
1 0.2 1 0.2 0.9 0.1 0.8 0.5


c)
MuV Enders
CMTR1 - + +
SAM - + -
IFIT1 - + - + - +
1 2 3 4 5 6
NP
1 0.2 1.1 0.6 1.1 0.2
d)
PIV5 W3
- + +
- + -
- + - + - +
1 2 3 4 5 6
NP
M
1 0.4 0.9 1 1.2 0.5
e)
PIV5 W3
NP
M
1
2
NP
M
3
4