Sapovirus Translation Requires an Interaction Between VPg and the Cap Binding Protein eIF4E

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ABSTRACT

Sapoviruses of the *Caliciviridae* family of small RNA viruses, are emerging pathogens that cause gastroenteritis in humans and animals. Molecular studies on human sapovirus have been hampered due to the lack of a cell culture system. In contrast, porcine sapovirus (PSaV) can be grown in cell culture making it a suitable model for understanding the infectious cycle of sapoviruses and related-enteric caliciviruses. Caliciviruses are known to use a novel mechanism of protein synthesis that relies on the interaction of cellular translation initiation factors with the virus encoded VPg protein that is covalently linked to the 5' end of the viral genome. Using PSaV as a representative member of the *Sapovirus* genus, we characterized the role of the viral VPg protein in sapovirus translation. As observed for other caliciviruses, the PSaV genome was found to be covalently linked to VPg and this linkage was required for the translation and the infectivity of viral RNA. The PSaV VPg protein was associated with the eIF4F complex in infected cells and bound directly to the eIF4E protein. As have been previously demonstrated for feline calicivirus, a member of the *Vesivirus* genus, PSaV translation required eIF4E and the interaction between eIF4E and eIF4G. Overall our study provides new insights into the novel mechanism of sapovirus translation suggesting that sapovirus VPg can hijack the cellular translation initiation mechanism by recruiting the eIF4F complex through a direct eIF4E interaction.

IMPORTANCE

Sapoviruses, of the *Caliciviridae* family, are one of the causative agents of viral gastroenteritis in humans. However, human sapovirus remains non cultivable in the cell culture hampering the ability to characterize the virus infectious cycle. Here, we show that the VPg protein from porcine sapovirus, the only cultivatable sapovirus, is essential for viral translation and functions via a direct interaction with the cellular translation initiation factor eIF4E. This work provides new insights into the novel protein primed mechanism of calicivirus VPg-dependent translation initiation.

INTRODUCTION

Sapoviruses belong to the *Caliciviridae* family and are recognized as cause of acute gastroenteritis worldwide (1–3). Based on the complete capsid sequence, sapoviruses are divided into five genogroups (GI-GV). Genogroups I, II, IV and V are known to infect humans, whereas the PSaV belongs to genogroup III. An increasing prevalence of sapovirus infections has been described highlighting their emerging role as a public health problem (2, 4). Despite this, limited studies on molecular mechanisms of the sapovirus infectious cycle have been conducted due to the lack of a cell culture system. PSaV, the prototype of the genus, has a permissive culture system (5, 6), making it a suitable model for understanding the

infectious cycle and molecular mechanisms of sapovirus translation and replication.

Sapoviruses have a small single-strand positive-sense RNA genome of approximately 7.3 to 7.5 kb, predicted to contain two or three open reading frames (ORF) (7, 8). ORF1 encodes 7 non-structural proteins (NS), and the major capsid protein (VP1). The NS5 region encodes the VPg protein, which in other caliciviruses has been found to be covalently linked to the 5' terminal of murine norovirus (MNV) and feline calicivirus (FCV) viral RNA (9, 10). PSaV ORF2, the equivalent of the FCV ORF3, is thought to encode a minor structural protein that by analogy with other caliciviruses may also play are role in viral replication (11, 12). Sapovirus ORF3 is present in some strains of the virus but function is as yet to be known (7).

As with all positive sense RNA viruses, sapovirus translation initiates immediately upon cell entry with the viral genome acting as an mRNA template. The expression of viral proteins is frequently subject to regulation at the level of the initiation of mRNA translation. There are at least 12 eukaryotic translation initiation factors (eIF) recruited during the initial stage of protein synthesis (13). Among these, a cap-binding complex (eIF4F) binds to the 5' end of the cellular mRNAs and recruits other factors to form a highly stable ribonucleoprotein complex for protein synthesis (13, 14). eIF4F is a heterotrimeric complex consisting of a cap binding protein eIF4E, scaffold protein eIF4G and an RNA helicase eIF4A. During the translation initiation process, eIF4E binds to the 7-

methyguanosine cap structure of the host mRNA and then recruits and activates eIF4G and eIF4A. eIF4A unwinds the mRNA at the 5' end and facilitate ribosome binding (13). eIF4G acts as the cornerstone for the multi-subunit eIF4F complex linking mRNA cap and ribosomal subunit via eIF4E and eIF3 binding domains respectively (15).

Many RNA viruses have developed unique mechanisms to usurp the host cell translation machinery for viral protein synthesis. Cap-independent translation is well characterized in the *Picornaviridae*, where internal ribosomal entry site (IRES)-mediated translation is used (16). Recently, another cap-independent translation mechanism primed by a viral encoded protein has been characterized in caliciviruses. Like picornavirus, calicivirus genomes do not possess a 5' cap structure; instead a viral protein genome (VPg) is covalently linked to the 5' end of the genome (17, 18). Recent studies have demonstrated a critical role for VPg in caliciviruses viral protein synthesis (9) as treatment of FCV or MNV VPg-linked RNA with proteinase K abrogated translation and infectivity (9, 10). The Norwalk virus VPg protein also interacts with eIF3 (19, 20). FCV VPg-dependent translation is directly coupled with the interaction of VPg with the cap binding protein eIF4E and eIF4E is functionally required for FCV translation (9). MNV translation is not affected by depletion of eIF4E nor by the separation of the eIF4E binding domain from eIF4G, but shows a functional requirement for eIF4A (9). This suggests that within the *Caliciviridae* family, the functional requirements for eIF4F components differ (9, 21–23).

105 The mechanism of translation initiation used by sapoviruses is currently
106 unknown. Therefore to characterise the role of sapovirus VPg in initiation of viral
107 protein synthesis, we performed a series of biochemical and *in vitro* studies. We
108 have demonstrated that the linkage of VPg to the PSaV genome is required for
109 the translation and infectivity of viral RNA. We also show a clear role for a VPg-
110 eIF4E interaction in PSaV translation indicating that PSaV is functionally similar
111 to FCV. These results contribute to a more detailed understanding of sapovirus
112 translation and the role of PSaV VPg protein during initiation of viral protein
113 synthesis.

114

115 **MATERIALS AND METHODS**

116

117 **Virus, cells and reagents**. The PSaV Cowden strain was obtained from Dr. K.O.
118 Chang (Kansas State University) and is a tissue culture adapted strain recovered
119 from the full-length infectious clone pCV4A (24). PSaV permissible LLC-PK1 cells
120 were transduced with lentiviruses expressing the bovine viral diarrhea disease
121 virus (BVDV) Npro protein to produce an IFN-deficient cell line, which allows
122 more efficient virus replication (M.Hosmillo, F. Soorgeloos, R. Hiraide,
123 I.Goodfellow and K-O. Cho, submitted for publication). IFN-deficient LLC-PK1
124 derived transduced cells expressing BVDV NPro (LLC-PK-Npro) were used to
125 propagate the PSaV in Eagle's minimal essential medium (EMEM) supplemented

126 with 200 μM glycochenodeoxycholic acid (GCDCA, Sigma), 2.5% fetal calf serum
127 and 1% penicillin/streptomycin (P/S) at 37°C with 5% CO_2.
128
129 Antisera to PSaV VPg and capsid were generated by immunization of New
130 Zealand white rabbits with purified recombinant VPg and virus, respectively.
131 Antisera against eIF4E, eIF4A and eIF4G1 were purchased from Cell Signaling
132 Technology.
133
134 Cap analogue (m7 G(5')ppp(5')G, Promega), elastatinal (Calbiochem),
135 proteinase K (Ambion) and [^{35}S] Methionine (PerkinElmer) were purchased.
136 Recombinant 4E-BP1 was a kind gift from Simon Morley (University of Sussex)
137 and the recombinant FMDV Lb protease was provided by Tim Skern (University
138 of Vienna). The eIF4E expression plasmid was the kindly received from Stephen
139 Curry (Imperial College London). Recombinant eIF4E was first purified with His
140 tag by affinity chromatography on a Hitrap chelating column (GE Healthcare). To
141 ensure a functional eIF4E protein, purified eIF4E was then additionally purified
142 via m7-GTP Sepharose column (GE Healthcare) using a salt gradient elution to
143 avoid possible contamination with cap analogue (9).
144
145 **Expression and purification of recombinant PSaV VPg**. The cDNA encoding
146 PSaV VPg was PCR amplified from a full-length clone pCV4A using primers
147 VPg-F (5'- GCG ACC ATG GCG AAA GGG AAA AAC AAA CGC) and VPg-R (5'

-TTA CTC GAG TCA CTC ACT GTC ATA GGT GTC ACC). PCR amplicons were cloned into pProEX HTc digested with NcoI and XhoI (underlined) and the resulting constructs were verified by sequencing.

VPg containing plasmid was expressed in B834 (DE3) *E. coli* strain. A large scale recombinant protein production was performed in an autoinducible ZYP media containing antibiotics, and cultured for 2 d at 37°C with shaking. Cell pellets from the bacterial culture were resuspended in a lysis buffer containing 20 mM Tris-HCl pH 7.5 and 500 mM NaCl, and one tablet of EDTA-free protease inhibitor (Roche). Following resuspension, the pellets were digested and sonicated. The lysates were collected and loaded onto a 5 mL HisTRAP HP column (GE Healthcare) and His-tagged VPg proteins were eluted in 500 mM imidazole. To prepare the untagged VPg protein, His-tag from VPg were digested by tobacco etch virus protease (kindly provided by Jeong-sun Kim, Chonnam National University) at 18°C overnight and purified again by HisTrap HP column. The final recombinant untagged VPg protein was concentrated and snap frozen in liquid nitrogen before storage at -80°C.

VPg-dependent *in-vitro* translation. Mock or PSaV were inoculated in LLC-PK-Npro cells at m.o.i. of 10 TCID50/cell. At 12, 24 and 48 h post infection, cells were harvested and total RNA were extracted using GenElute total RNA extraction kit (Sigma). *In vitro* translation reactions using Flexi rabbit reticulocyte

lysate (RRL, Promega) was performed as previously described (9) using 40 µg ml^{-1} mock or PSaV RNA and 12.5 µg ml^{-1} *in vitro* transcribed control RNAs. Capped control dicistronic mRNAs containing either foot and mouth disease virus (FMDV) or porcine teschovirus-1 (PTV)-IRES were synthesized *in vitro* from plasmids pGEM-rluc/FMDV/fluc or pGEM-CAT/PTV/LUC provided by Graham Belsham (Technical University of Denmark) respectively (25). Control capped PSaV RNA were *in vitro* transcribed from pCV4A containing the full-length PSaV genome. Transcribed RNAs were then capped using ScriptCap m7-G capping system (Epicentre Biotechnologies).

In reactions that required the addition of cap analogue, 4E-BP1, Lb protease and eIF4E, recombinant proteins were preincubated at 30°C for 15 min prior to the addition of RNA. Complementation with recombinant eIF4E after eIF4E sequestration or depletion was carried out for additional 5 min before incubation of the RNA template. After the incorporation of RNA, *in vitro* translation was performed at 30°C for 90 min and terminated with equal volume of tris buffer containing 10 mM EDTA and 100 ng ml^{-1} RNASe A (Ambion). Translated proteins were resuspended in 5x SDS sample buffer and resolved on 12.5% polyacrylamide gels. Pretreatment of RNAs was carried out by incubation of RNA in 10 mM Tris, pH 8.0, 1.0 mM EDTA, 0.1M NaCl and 0.5% SDS in the presence or absence of 10 µg ml^{-1} proteinase K for 30 min at 37°C. Pretreated RNAs were immediately purified by GenElute RNA clean up protocol (Sigma). Protein synthesis levels were quantitated from dried gels using a Packard Instant Imager

(Canberra, Packard, UK). X-ray films were developed after 4-24 h incubation at room temperature.

Immunoprecipitation. Products from IVT reactions were incubated with protein A agarose beads and antibodies against MNV NS7, PSaV VPg or VP1 in radioimmunoprecipitation assay buffer (RIPA, 50 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) buffer. The mixture was centrifuged, washed with RIPA buffer 3 times and the immunoprecipitated proteins were then evaluated by autoradiography.

Avicel-based plaque assay. Total RNA extracts (10 μg) from mock or PSaV infected cells were pretreated with and without proteinase K as above. Treated or mock-treated RNA were transfected into LLC-PK-Npro using lipofectamine 2000 as per the manufacturer's instructions (Invitrogen). After 4 h, the cells were washed and overlaid with 1.3% Avicel cellulose (FMC Health and Nutrition) in EMEM supplemented with 2.5% FBS, 0.225% sodium bicarbonate and 1.0% penicillin/streptomycin. Plates were incubated at 37°C for 4 days. After incubation, the Avicel mixture was removed, and cells were fixed and stained with 1.6% methylene blue and with 33.3% formaldehyde solution in 1x PBS for 30 min. Plates were washed with distilled water until the desired staining intensity was achieved. Infectious virus titer was quantified by plaque assay method.

214 **Cap-sepharose purification for eIF4F complex**. Cell lysates were prepared
215 from either mock or PSaV infected (m.o.i of 10 TCID50/cell) LLC-PK-Npro cells in
216 cap-sepharose lysis buffer (100 mM KCl, 0.1 mM EDTA, 10% glycerol, 2 mM
217 $MgCl_2$, 20 mM HEPES pH 7.6) and ribonuclease treated for 15 min at room
218 temperature. Cytoplasmic extracts were centrifuged and incubated with m7-GTP
219 sepharose (GE Healthcare) overnight at 4°C. eIF4F enriched complex was
220 precipitated and washed 2 times with lysis buffer. Bound proteins were eluted in
221 2x reducing buffer, resolved by SDS-PAGE and analysed by immunoblot.
222

223 **ELISA-based initiation factor capture assay**. An ELISA-based capture assay
224 was performed as described previously (26). Briefly, purified PSaV VPg (1 μg)
225 was coated onto 96-well plates (NUNC immunosorb) overnight at 4°C. The wells
226 were then blocked with 2% BSA in tris-buffer saline (TBS). Hela cell lysates or
227 recombinant eIF4E were diluted in 2% BSA in TBS with 0.05% Tween-20 and
228 was incubated for 2 h at 4°C with previously coated wells. The association to
229 eIF4E, eIF4A and eIF4G was detected using specific primary antibodies.
230 Secondary HRP conjugated antibodies were added, and signals were developed
231 with TMB substrate. Reaction was stopped with H_2SO_4 and the optical density
232 was determined at 450 nM.
233

234 **Pull down assay**. His-tagged PSaV VPg was immobilized on HisPur cobalt resin

(Pierce) and incubated with either recombinant eIF4E or ribonuclease-treated cell lysates on the rotator for 4 h to overnight at 4°C, respectively. Proteins interacting with His-tagged VPg were pulled down by centrifugation and sequentially washed 3 to 5 times in lysis buffer. Bound proteins were eluted with 400 mM imidazole. Binding to eIF4F components or recombinant eIF4E was analysed by western blot.

eIF4E siRNA-based functional assay. Confluent cell culture of LLC-PK-Npro were transfected with either siRNA against non-specific target (100 pmol) or eIF4E (100 pmol) using Lipofectamine 2000 following manufacturer's instructions. Cells were then infected at an m.o.i. of 1 TCID50/cell. Unabsorbed viruses were removed after 4h, and infected cells were maintained in EMEM with 2.5% FBS and 200 μM GCDCA. The virus titer was analysed 24 h post infection by RT-qPCR using specific PSaV primers targeting viral protease, 5'-CAACAATGGCACAACAACG-3' (forward) and 5'-ACAAGCTTCTTC ACCCCACA-3' (reverse).

RESULTS

The porcine sapovirus RNA is linked to VPg. Based on data obtained on other genera of the *Caliciviridae*, sapovirus RNA is predicted to be VPg-linked (Fig. 1A). To date, however, no experimental evidence for the formation of VPg-linked sapovirus RNA has been described. To determine if VPg is linked to the PSaV genome, we examined lysates and purified RNAs from infected LLC-PK-Npro cells for the presence of VPg by western blot. It is important to note that we used interferon deficient LLC-PK-Npro cells to infect mock or PSaV for total RNA extractions to enable us to harvest the sufficient amount of viral RNA that facilitate an *in vitro* translation reaction. Using this cell line, our previous studies showed a 100-fold increase in virus replication and thus, these cells were consequently used for virus propagation (M.Hosmillo, F. Soorgeloos, R. Hiraide, I.Goodfellow and K-O. Cho, submitted for publication). This cell line shall be referred to as LLC-PK-Npro throughout the manuscript.

Cell lysates harvested from PSaV infected cells showed the presence of a fully processed form of VPg (15 kDa) and two VPg containing precursors from polyprotein which based on their molecular weights, corresponded to NS4-VPg (46 kDa) and NS4-VPg-NS6-7 (85 kDa) (Fig. 1B). To determine which form of VPg is linked to the viral genome, we examined purified RNA isolated from mock and PSaV infected cells for the presence of VPg after ribonuclease treatment. western blot analysis revealed that only the fully processed form of VPg is linked

to PSaV genome (Fig. 1B) as observed for MNV (9). This confirms that like the other *Calicivirus* genera, the genome of PSaV, a member of the *Sapovirus* genus is also covalently linked at the 5' end.

Porcine sapovirus VPg is required for infectivity and the translation of viral RNA. To characterize the translation initiation of PSaV, RRL were used to examine the ability of the VPg-linked RNA to translate *in vitro*. Total RNA was extracted from either mock or PSaV infected cells and used to program RRL for *in vitro* translation reactions. The translation profile from PSaV RNA showed several additional proteins compared with that of RNA from mock infected cells (Fig. 2A). These additional proteins were predicted to be of viral origin as the viral RNA used to produce these proteins increased during the course of infection. To determine if the additional protein products were in fact PSaV specific, immunoprecipitations were performed using antisera specific to several PSaV proteins. Using antibodies against the PSaV VPg and capsid protein (VP1), proteins corresponding to predicted molecular weights of VP1 and precursor forms of VPg were immunoprecipitated (Fig. 2B). As expected, no significant levels of PSaV viral proteins were immunoprecipitated with antisera to the MNV NS7 protein, although a small amount of the capsid protein was often detected due to the high levels of expression (Fig. 2B).

294 To determine if PSaV translation is VPg-dependent, we examined the effect of
295 proteinase K (Pk) treatment on the translation profile of RNA. As controls, we
296 used RNAs isolated from either mock infected cells and capped *in vitro*
297 transcribed RNA of a dicistronic construct, expressing the CAT protein in a cap-
298 dependent manner and the fluc protein dependent on FMDV-IRES structure. In
299 addition, we used capped *in vitro* transcribed full-length PSaV RNA. Following Pk
300 treatment, RNA samples were confirmed to be intact before subjecting to *in-vitro*
301 translation and transfection (Fig. 2C). The translation of VPg-linked PSaV RNA
302 was abrogated by prior treatment with Pk (Fig. 2D), confirming that VPg linkage
303 is necessary for viral translation *in vitro*. To further confirm the role of VPg in
304 PSaV translation during replication in cells, we then examined the effect of Pk
305 treatment on PSaV RNA infectivity. We measured the specific infectivity of Pk or
306 mock treated PSaV RNA in permissive LLC-PK1 cells. Without Pk pretreatment,
307 PSaV RNA yielded up 1.6 x 10^5 pfu/µg of total RNA but this was markedly
308 reduced to 8 pfu/µg after Pk treatment (Fig. 2E).
309
310 **Porcine sapovirus VPg is associated with cellular translation initiation**
311 **factors during virus infection.** Previous studies on FCV and MNV translation
312 revealed the association of VPg with cellular translation initiation factors during
313 viral replication in cell culture (9, 10). Given our observation that PSaV VPg was
314 required for viral translation and infectivity, this also suggested that PSaV VPg
315 was associated with the cellular translation machinery. To investigate if the PSaV

316 VPg protein interacted with the translation initiation factor complex (eIF4F), we
317 purified the eIF4F complex from mock and infected cells using m7-GTP
318 sepharose resin, which binds to eIF4E, and therefore can be used to enrich
319 eIF4F-interacting proteins. The eIF4E containing complex isolated from infected
320 cells also contained PSaV VPg, both as precursor and mature forms (Fig. 3A). As
321 expected, the eIF4E protein was isolated by m7-GTP sepharose whereas
322 GAPDH was not, confirming the specificity of the assay (Fig. 3A). To examine the
323 interaction of PSaV VPg and eIF4F complex from the cell lysates, recombinant
324 His-tagged VPg protein was incubated with nuclease-treated cell lysates and
325 purified using cobalt resin. Western blot analysis showed that the components of
326 eIF4F were pulled down with His-tagged VPg, but not with BSA (Fig. 3B). No
327 interaction between VPg and GAPDH was observed, verifying the specificity of
328 the pull down assay. To further confirm the association of VPg with components
329 of the eIF4F complex, a capture ELISA-based binding assay was performed
330 whereby wells were coated with recombinant PSaV VPg or BSA and then
331 incubated with lysates from a permissive cell. The retention of the eIF4F complex
332 was then examined by ELISA using anti-initiation factor antibodies. VPg retained
333 the eIF4E, 4A and 4G components of the eIF4F complex confirming the
334 association of PSaV VPg with these cellular translation initiation factors (Fig. 3C).
335 As expected, control wells coated with BSA did not show any interaction with the
336 eIF4F components (Fig. 3C).
337

Porcine sapovirus VPg binds to recombinant eIF4E directly. To determine if VPg interacted directly with eIF4E, as observed for other caliciviruses, an ELISA-based capture assay was performed. Plates coated with PSaV VPg, but not BSA, showed a direct binding with purified recombinant eIF4E in dose dependent manner (Fig. 4A). To confirm this interaction, His-tagged PSaV VPg protein was immobilized on cobalt resin and incubated with recombinant eIF4E in a pull down assay. Western blot analysis further demonstrated direct interaction between PSaV VPg and eIF4E, but no interaction was found in resin treated with BSA as a control (Fig. 4B).

Porcine sapovirus translation is insensitive to cap analogue but requires an eIF4E-4G interaction. To further examine the functional requirements of PSaV RNA translation, RRL was used as an experimental system to probe the relative roles of eIF4E and the eIF4E-eIF4G interaction. To determine whether or not the interaction of eIF4E with cap analog inhibits PSaV translation, the effect of increasing concentrations of cap analogue on PSaV translation was examined. As expected, cap analogue inhibited cap-dependent translation, but had no effect on FMDV IRES-dependent translation (Fig. 5A). In contrast, PSaV translation was unaffected, suggesting that there are distinct sites for VPg and cap binding to eIF4E, fitting with our ability to co-purify VPg on m7-GTP sepharose (Fig. 3A). In addition, when eIF4E was sequestered by the addition of recombinant eIF4E binding protein (4E-BP1), translation of PSaV RNA was reduced, as was cap-

dependent translation (Fig. 5B). As expected, 4E-BP1 had no effect on PTV IRES-mediated translation (Fig. 5B). The 4E-BP1 mediated inhibition of PSaV and cap-dependent translation could be restored by the addition of recombinant eIF4E confirming the specificity of the inhibitory effect of 4E-BP1 on eIF4E (Fig. 5C).

Porcine sapovirus translation requires an intact eIF4G. To examine the role of the eIF4E-4G interaction and to examine if the N-terminal eIF4E binding domain of eIF4G is required for PSaV translation, RRL was pretreated with FMDV Lb protease, which cleaves eIF4G separating the eIF4E-binding domain from eIF4A, PABP and eIF3 binding domains (Fig. 6A). Cleavage of eIF4G was verified by western blot analysis, showing increasing eIF4G cleavage with higher concentrations of Lb protease (Fig. 6B). Cleavage of eIF4G reduced translation of PSaV RNA and cap-dependent translation, whereas PTV IRES-dependent translation was slightly increased (Fig. 6C). A protein translated from PSaV RNA preparations corresponding to a host mRNA (highlighted with an asterisk in Fig. 6C) was inhibited by eIF4G cleavage.

Porcine sapovirus translation is sensitive to eIF4E depletion. To further examine the role of the PSaV VPg-eIF4E interaction, the effect of eIF4E depletion on PSaV translation was investigated. RRL was depleted of eIF4E using m7-GTP sepharose after the addition of 4E-BP1 to prevent the concomitant

382 removal of eIF4G. Depletion of eIF4E was confirmed by western blot analysis.
383 eIF4E was significantly depleted, whereas the levels of eIF4A and eIF4G
384 remained largely similar to mock depleted lysate (Fig. 7A). Cap and PSaV-VPg
385 dependent translation were inhibited by eIF4E depletion, however PTV IRES-
386 mediated translation was slightly stimulated (Fig. 7B). The addition of
387 recombinant eIF4E restored cap dependent and PSaV VPg-dependent
388 translations to levels similar to those observed in mock depleted lysates (Fig.
389 7B).
390
391 **Porcine sapovirus requires eIF4E in the cell culture**. RNA interference was
392 used to deplete eIF4E and the effect on PSaV replication was examined. The
393 expression of eIF4E was confirmed after double transfection of siRNA against
394 eIF4E (Fig. 8A), showing a significant reduction in eIF4E expression.
395 Subsequently, inoculation of PSaV showed an eIF4E siRNA-mediated inhibition
396 of virus replication. PSaV mRNA levels were significantly reduced in cells
397 transfected with eIF4E siRNA (Fig. 8B).

DISCUSSION

In the current study, we have demonstrated that the PSaV VPg protein is associated with the eIF4F translation initiation complex facilitating viral protein synthesis. Our data fits with previous studies on other member of the *Caliciviridae* indicating that VPg functions as proteinaceous cap substitute (9, 10, 20). This novel mechanism of protein-primed translation initiation is also found in members of *Potyviridae* family of RNA viruses that infect plants (27, 28). Recent studies also indicate that members of *Astroviridae* possess a genome-linked VPg protein and the linkage of VPg to the viral RNA is essential for infectivity (29), although whether in this instance, VPg contributes to viral translation has yet to be determined. In the case of potyviruses, the interaction of VPg with eIF(iso)4E is a major determinant of a host susceptibility whereby mutations in eIF(iso)4E lead to resistance to infection (30).

Our data indicates that the recruitment of eIF4F complex to the 5' end of PSAV RNA occurs via a direct VPg-eIF4E interaction and that this interaction occurs independently of the cap-binding site on eIF4E. In contrast, the interaction of potyvirus VPg with eIF4E competes for cap binding and can inhibit host cell translation (31). Within the *Caliciviridae* family, the interaction of VPg with eIF4E appears to be conserved, as similar VPg-eIF4E interactions have been described in FCV, a member of the *Vesivirus* genus, and MNV, a member of the *Norovirus* genus (9, 10). Importantly, whilst the interaction of the FCV VPg protein with eIF4E is essential for viral translation, the MNV VPg-eIF4E interaction does not

appear to contribute to viral protein synthesis and may play a secondary role in the modulation of eIF4E activity. In the case of MNV, we have recently identified a direct VPg-eIF4G interaction that is essential for MNV translation initiation (32). Our data would indicate that PSaV VPg-dependent translation is functionally identical to that of FCV in that a VPg-eIF4E interaction is essential, as is the interaction of eIF4E with eIF4G. The functional requirements and possible interaction of VPg with other components of the eIF4F complex is the focus of on-going studies.

With the data presented in the current study, functional information on the relative requirements of some of the eIF4F components for three of the five genera of the *Caliciviridae* family, namely *Vesivirus, Norovirus* and *Sapovirus*, have now been described. However, further studies will be required to unravel the mechanism of translation used by members of *Lagovirus* and *Nebovirus* genera. In the case of lagoviruses, reverse genetics studies would indicate that uncapped *in vitro* transcribed RNA is infectious when transfected into immortalized cells, indicating that VPg is not essential for the initiation of infection (33). Therefore, whilst the interaction of translation initiation factors with the VPg proteins of caliciviruses is a conserved feature, subtle differences in the functional requirements of initiation factors apparent between genera. Similar observation have been made in the study of IRES-mediated translation of the *Picornaviridae*; for example, the hepatitis A virus IRES requires eIF4E and an intact eIF4G, whereas that of

poliovirus and FMDV does not require eIF4E and can function using a cleaved form of eIF4G (34, 35). The biological relevance of the subtle differences in initiation factor requirements for calicivirus translation has yet to be determined, but may in some way have occurred as a consequence of the variation in pathogenesis, i.e. FCV and PSaV typically cause acute self-limiting infection in the natural host, whereas MNV usually causes long-term persistent infection. These differences in the nature of the disease may be at least partially due to a consequence of differences in the translation efficiency of viral RNA in cells, although further comparative studies on the efficiency of translation area required. Fitting with this hypothesis, the PSaV VPg protein shares a higher degree of sequence similarity with FCV than MNV.

In summary, our study provides additional insights into the novel mechanism of protein-primed VPg-dependent translation initiation used by members of the *Caliciviridae* family of small positive sense RNA viruses. This work also highlights how viruses have often evolved novel mechanisms to translate their mRNAs in the presence of high levels of competing cellular mRNAs.

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FIGURE LEGENDS

FIG 1 The porcine sapovirus genome is linked to mature viral protein-genome (VPg) at its 5' terminal. (A) A schematic presentation of the porcine sapovirus genome showing the non-structural (NS1-NS6-7) and structural (VP1 and VP2) proteins. (B) Monolayers of LLC-PK1 cells were either mock infected or infected with PSaV at a m.o.i. of 10 TCID50/cell. After adsorption, the media was replaced with EMEM containing GCDCA and FBS. Infection proceeded for 30 h, then cell lysates and purified RNA were prepared. Purified RNAs were subjected to ribonuclease treatment then cell lysates and treated RNA were subsequently

591 analysed by western blot.

592

593 **FIG 2** Porcine sapovirus requires VPg linkage to confer virus infectivity and viral
594 RNA translation. (A) Monolayers of LLC-PK1 cells were infected by either mock
595 or PSaV at m.o.i. of 10 TCID50/cell. Cells were harvested at 12, 24 and 36 h.p.i.,
596 lysed, and the total RNA extracted. RNA isolated from infected cells during the
597 time course were used to program *in vitro* translation reaction using rabbit
598 reticulocyte lysates (RRL). The translation products were analysed by SDS-
599 PAGE and the translation profiles were then evaluated by autoradiography.
600 Asterisk refers to the translated background proteins from RRL. (B) Proteins
601 produced by *in vitro* translation were immunoprecipitated using antibodies
602 against the murine norovirus NS7 protein, PSaV VPg and capsid proteins
603 overnight. Immonoprecipitates were washed 3 times with RIPA buffer and then
604 resolved by 12.5 % SDS-PAGE. Protein precipitates were analysed by
605 autoradiography. (C) RNA samples prepared from in vitro transcribed and
606 capped Cap-Rluc:FMDV IRES:Fluc dicistronic RNA, mock and PSaV-infected
607 cells and capped *in vitro* transcripts from the PSaV full length cDNA clone
608 pCV4A were pretreated with and without proteinase K at 37^{o}C for 30 min.
609 Following RNA purification, the purified RNA samples were analyzed by agarose
610 gel electrophoresis to confirm their integrity. (D) RNA samples were then used to
611 program RRLs and were subjected to an *in vitro* translation reaction. (E) RNA
612 extracted from PSaV infected cells, mock or proteinase K treated was transfected

613 into LLC-PK1 cells expressing BVDV Npro. Serial 10-fold dilutions of RNA
614 preparations were transfected and 4 d post transfection the cells were washed
615 and incubated in 1.3 % avicel-based overlay media containing 2.5 % FBS,
616 0.225% sodium bicarbonate and supplemented with 200 µm GCDCA. Cells were
617 immediately fixed and stained.

618

619 **FIG 3** The porcine sapovirus VPg binds to the cellular translation initiation factors
620 during virus infection. (A) Monolayers of LLC-PK1 cells transduced with BVDV
621 Npro were either mock infected or infected with PSaV at an m.o.i. of 10
622 TCID50/cells. 36 h post infection, cells were collected and lysed in cap-
623 sepharose buffer. Lysates were centrifuged and further treated with RNAse. One
624 thousand micrograms of lysates were incubated with cap-sepharose beads
625 overnight. After washing the bound proteins were analysed by SDS-PAGE and
626 western blot. (B) His-tag pull down assay was performed using 10 ug of His-
627 tagged VPg or BSA immobilized on HisPur cobalt resin. Increasing amounts (0-
628 50 ug) of nuclease-treated cell lysates were then incubated overnight with the
629 bait VPg protein. Protein complexes were extensively washed, eluted and
630 analyzed by western blot. (C) A capture ELISA was performed using 1 μg of
631 purified recombinant PSaV VPg or BSA as a control. Ten micrograms of
632 nuclease-treated cytoplasmic extracts were incubated with either target,
633 extensively washed prior to detection with rabbit antibodies to eIF4E, eIF4A or
634 eIF4G. Antibody binding was detected using a secondary anti-rabbit HRP

conjugated antibody followed by incubation with ELISA substrate. Samples were analysed in triplicate and in at least three independent experiments. One representative dataset is shown. Error bars represent standard deviation between the triplicated samples.

FIG 4 Porcine sapovirus VPg binds to recombinant eIF4E. (A) A capture ELISA was performed using 1μg of purified recombinant PSaV VPg or BSA as a control. Increasing concentrations of recombinant eIF4E were then incubated with both coated proteins and extensively washed prior to detection with rabbit antibodies to eIF4E. Antibody binding was detected using a secondary anti-rabbit HRP conjugated antibody followed by incubation with ELISA substrate. Samples were analysed in triplicate in at least three independent experiments. One representative dataset is shown. (B) A pull down assay was performed using 1 μg of recombinant His-tagged VPg immobilized on HisPur cobalt resin or resin alone. Resin with and without VPg were washed, blocked with BSA and incubated with similar concentration of either BSA or recombinant eIF4E. Binding to eIF4E was detected by immunoblot with anti-eIF4E antibody. Samples were analysed in duplicate and in at least three independent experiments and one representative dataset is shown.

FIG 5 Porcine sapovirus translation is independent on cap analogue but requires the eIF4E-4G interaction. (A) *In vitro* translation was performed as illustrated by the experimental time line using either VPg-linked PSaV RNA or dicistronic RNA containing a cap-dependent CAT and PTV IRES-dependent Rluc. Translation reactions were preincubated with increasing concentrations of CAP analogue, and then the RNAs were added to initiate the protein synthesis. Profiles for VPg-, cap- and IRES-dependent translations were resolved by SDS-PAGE page. The gels were fixed, dried and exposed to X-ray film. The intensity of each band was quantitated with reference to the value obtained in the absence of cap analogue. (B) *In vitro* translation was performed following the experimental scheme with increasing amounts of recombinant 4E-BP1, before addition of PSaV RNA and dicistronic RNA. RNAs were then added to initiate the protein synthesis. Profiles for VPg-, cap- and IRES-dependent translations were separated by SDS-PAGE. The gels were fixed, dried and exposed to X-ray film. The intensity of each bands were quantitated with reference samples incubated with 4E-BP1 buffer only. The asterisks represent the background signals from the host mRNA translation profile. (C) Experimental scheme of *in vitro* translation was performed as previously described with addition of recombinant eIF4E after sequestration by 4E-BP1. Effects of separation by 4E-BP1 buffer or 4E-BP1 and subsequent complementation of eIF4E in the cap-, VPg-, and IRES-dependent translation were evaluated by SDS-PAGE and autoradiograph.

676 **FIG 6** Porcine sapovirus translation requires full-length eIF4G. (A) *In vitro*
677 translation was performed following the experimental time-line using increasing
678 amounts of FMDV Lb protease to cleave eIF4G. Subsequently elastatinal was
679 added to quench the protease activity before the addition of PSaV RNA or *in vitro*
680 transcribed dicistronic RNA. (B) The effect of Lb protease was confirmed by
681 western blot using antibody against eIF4G. (C) Translation profiles for VPg-, cap-
682 and IRES-dependent expressing proteins were resolved by SDS-PAGE. The gels
683 were fixed, dried and exposed to X-ray film. The intensity of each bands were
684 quantitated with reference samples incubated without Lb protease. The asterisk
685 refers to the protein translated from PSaV RNA preparations corresponding to a
686 host mRNA which is also inhibited by eIF4G cleavage.

687

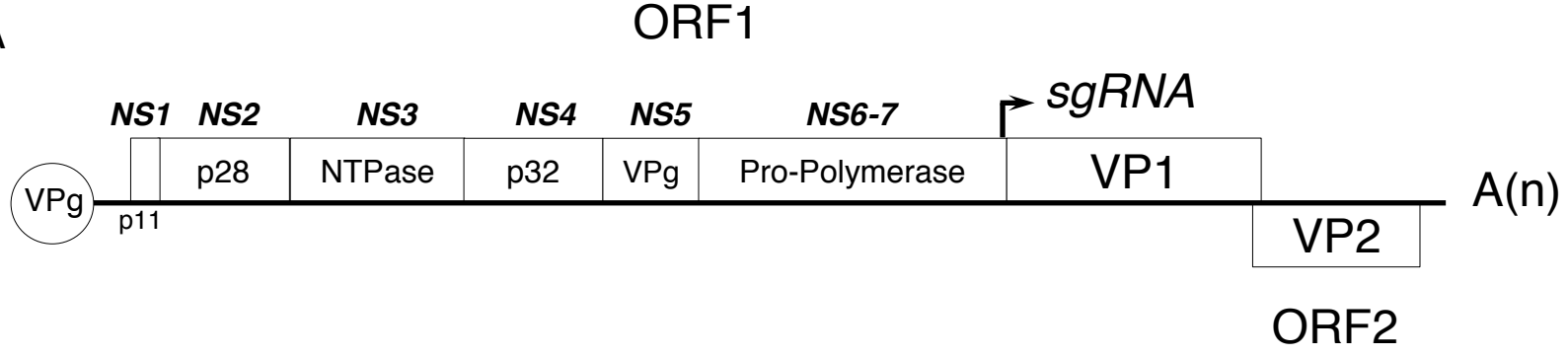
688 **FIG 7** Porcine sapovirus translation is sensitive to eIF4E depletion. Mock-
689 depleted or eIF4E-depleted RRL were used for *in vitro* translation reaction with
690 replenishment of recombinant eIF4E or buffer alone. (A) Depletion of eIF4E was
691 verified by western blot using 0.25, 0.5 and 1.0 μl of the RRL. (B) Translation of
692 VPg-, cap-, and IRES dependent proteins were observed in mock and eIF4E
693 depleted lysates, with and without addition of recombinant eIF4E.

694

695 **FIG 8** eIF4E is required for PSaV replication in the cell culture. (A) LLC-PK1 cells
696 were transfected with either control or eIF4E siRNAs as described in the

materials and methods. Reduced eIF4E expression was verified by western blot using antibody against eIF4E. (B) PSaV was then infected at m.o.i. of 0.2 TCID50/cell. Cells were harvested 24 h post infection. and RNA were extracted for qRT-PCR analysis targeting specifically to PSaV protease region. Samples were analysed in triplicate and in at least three independent experiments. Error bars represent standard error of the mean between the triplicated samples.

A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NS2
NS3
NS4
NS5
NS6-7
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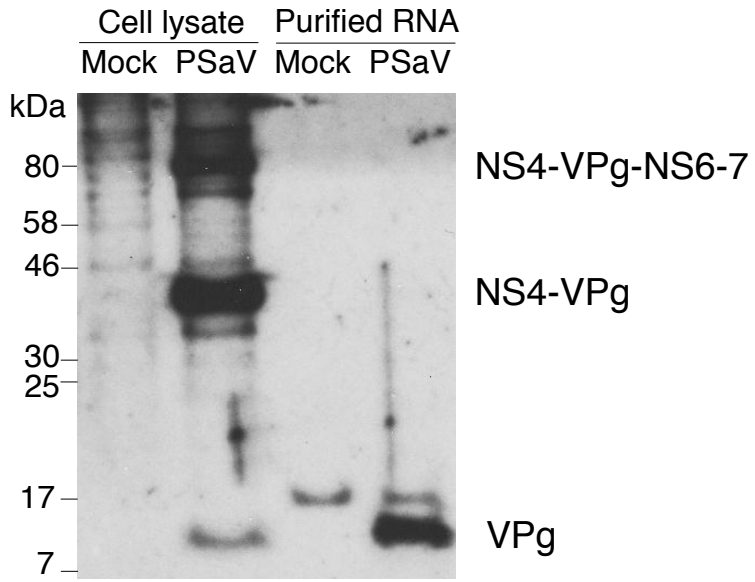
VP1
A(n)
VP2
ORF2

B


Cell lysate
Purified RNA
Mock
PSaV
Mock
PSaV
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46
30
25
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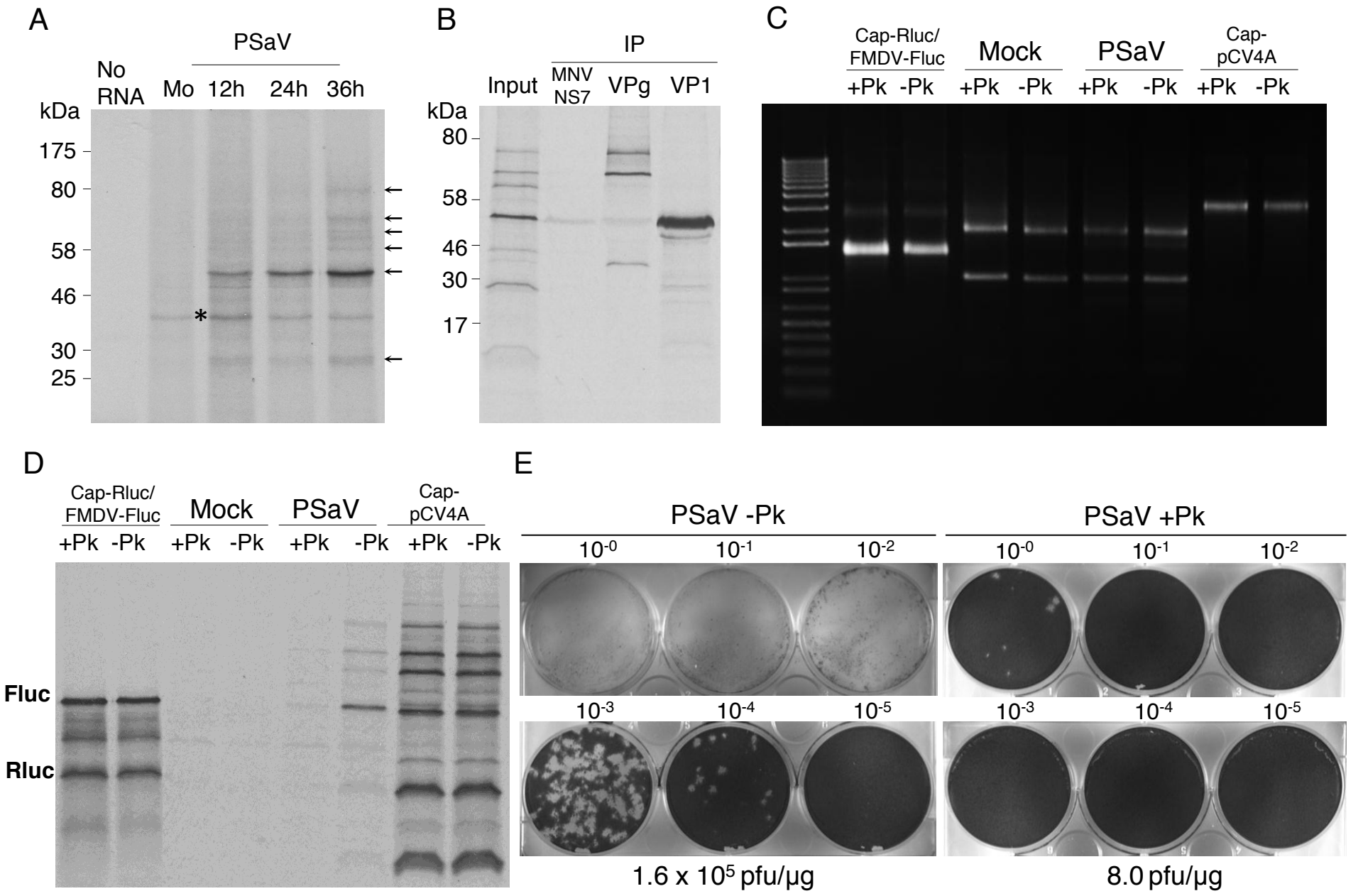
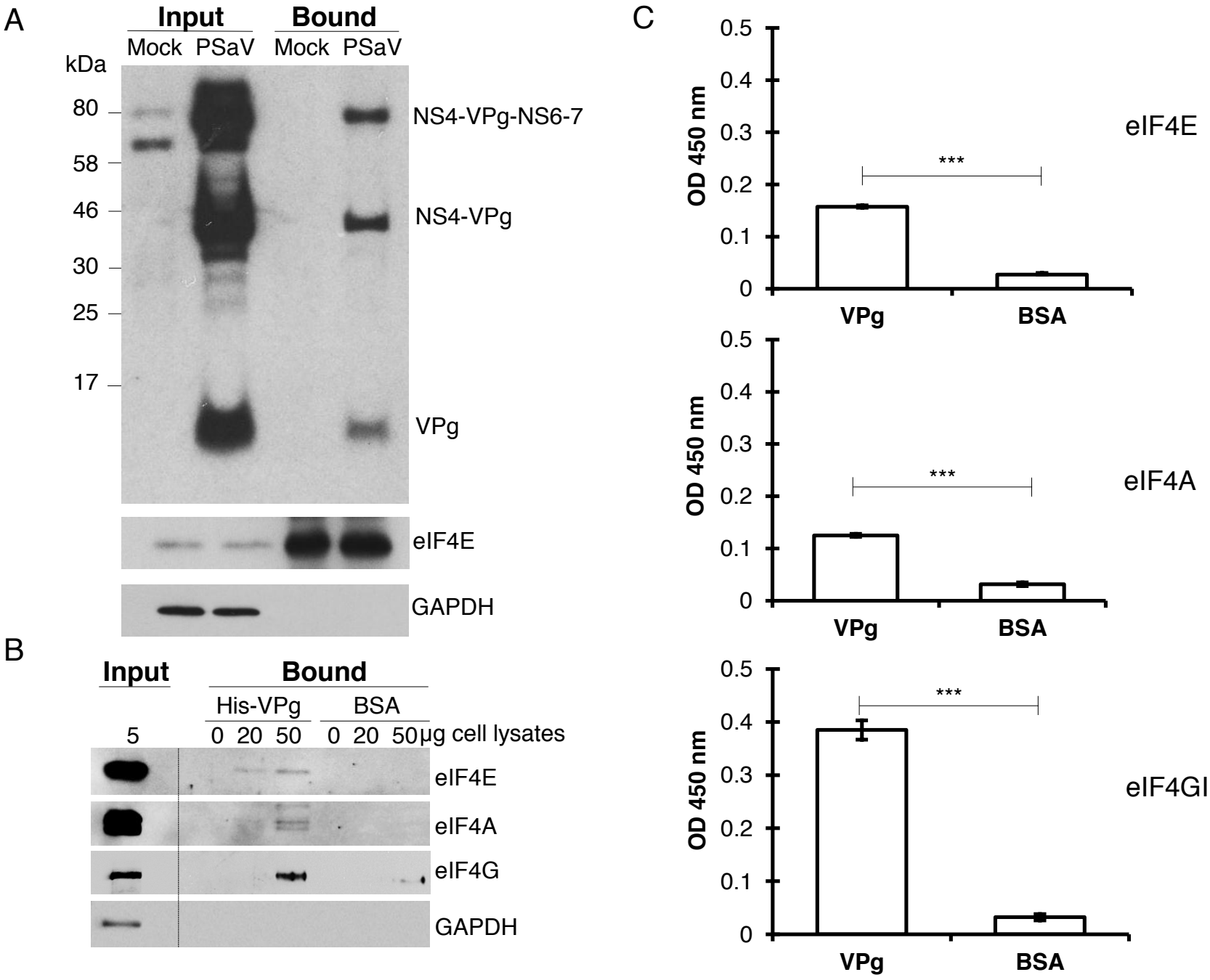
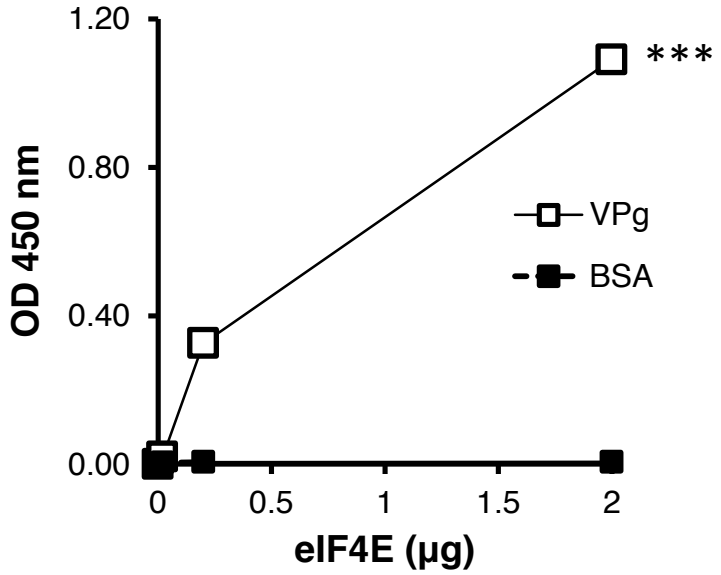
7
NS4-VPg-NS6-7
NS4-VPg
VPg

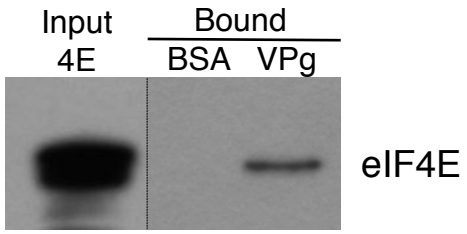

A
PSaV
No RNA
Mo
12h
24h
36h
kDa
175
80
58
46
30
25
*
B
IP
Input
MNV NS7
VPg
VP1
kDa
80
58
46
30
17
C
Cap-Rluc/ FMDV-Fluc
Mock
PSaV
Cap- pCV4A
+Pk -Pk +Pk -Pk +Pk -Pk +Pk -Pk
D
Cap-Rluc/ FMDV-Fluc
Mock
PSaV
Cap- pCV4A
+Pk -Pk +Pk -Pk +Pk -Pk +Pk -Pk
Fluc
Rluc
E
PSaV -Pk
PSaV +Pk
10-0 10-1 10-2
10-3 10-4 10-5
1.6 x 10^5 pfu/µg
8.0 pfu/µg


A
Input
Bound
Mock PSaV Mock PSaV
kDa
80
58
46
30
25
17
NS4-VPg-NS6-7
NS4-VPg
VPg
eIF4E
GAPDH
B
Input
Bound
His-VPg
BSA
5
0 20 50 0 20 50 µg cell lysates
eIF4E
eIF4A
eIF4G
GAPDH
C
OD 450 nm
0.5
0.4
0.3
0.2
0.1
0
***
VPg
BSA
eIF4E
eIF4A
eIF4GI

A


1.20
0.80
0.40
0.00
OD 450 nm
0
0.5
1
1.5
2
eIF4E (µg)
***
VPg
BSA

B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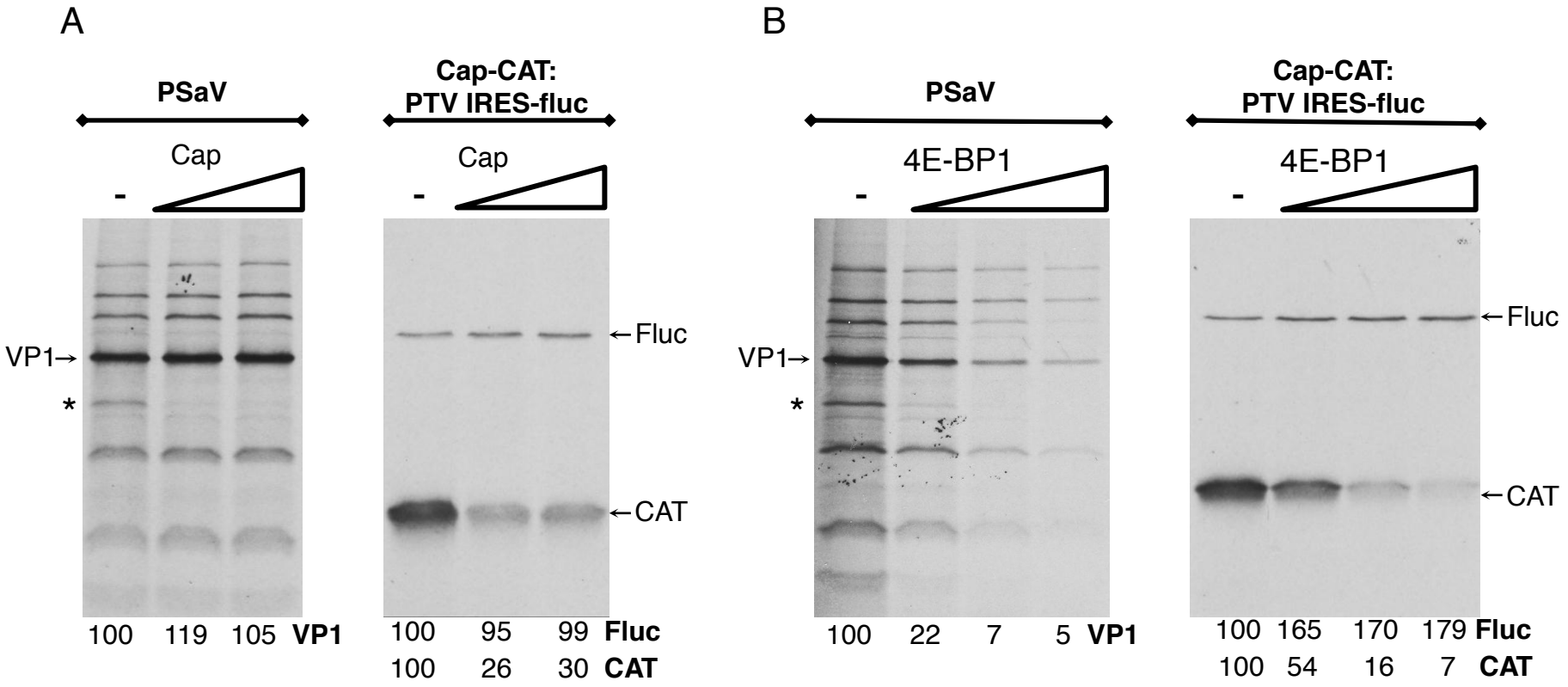
Bound
BSA
VPg
eIF4E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PSaV
Cap
-
Cap-CAT:
PTV IRES-fluc
Cap
-
VP1→
*
←Fluc
←CAT
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100 95 99 Fluc
100 26 30 CAT
B
PSaV
4E-BP1
-
Cap-CAT:
PTV IRES-fluc
4E-BP1
-
VP1→
*
←Fluc
←CAT
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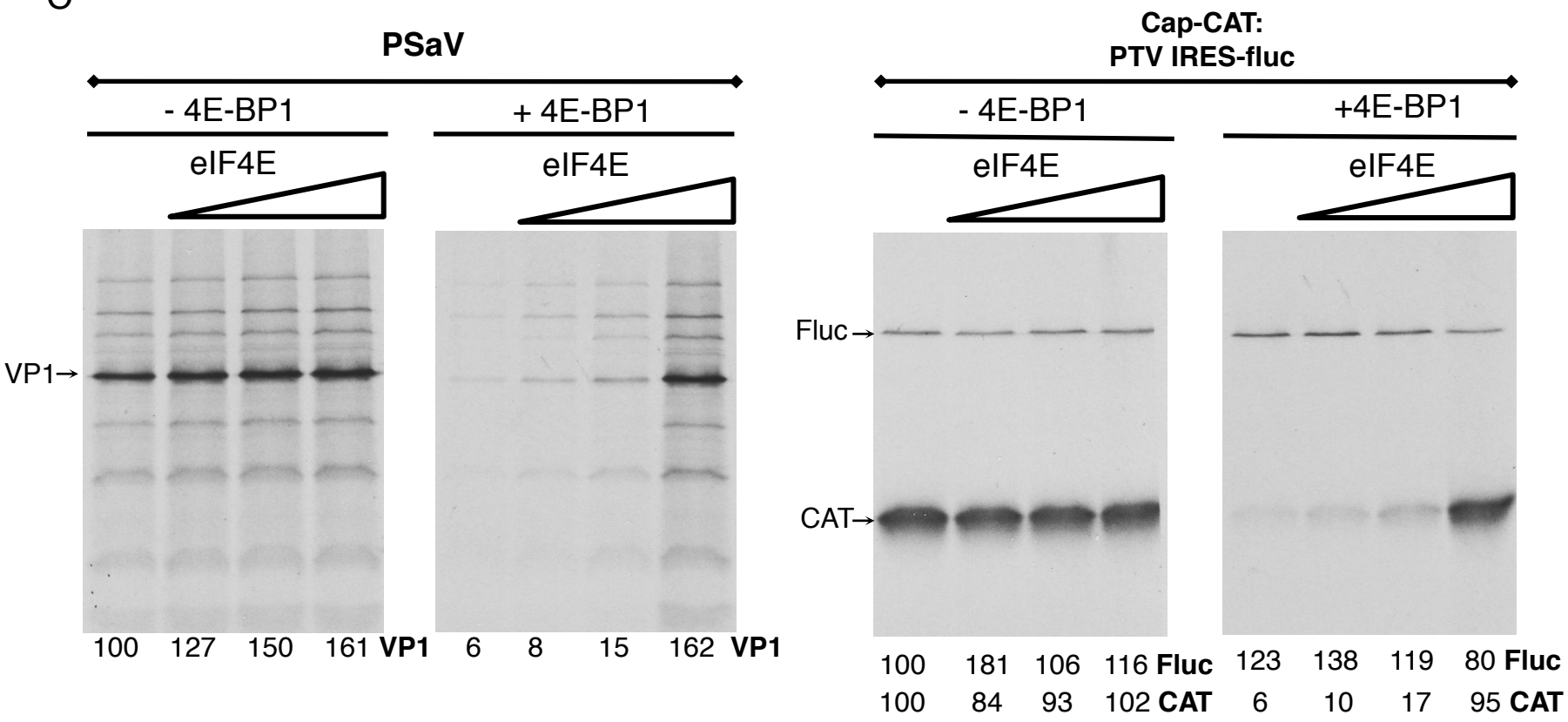
100 165 170 179 Fluc
100 54 16 7 CAT

C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+ 4E-BP1
eIF4E
eIF4E
VP1→
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6 8 15 162 VP1
Cap-CAT:
PTV IRES-fluc
- 4E-BP1
+4E-BP1
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CAT→
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100 84 93 102 CAT
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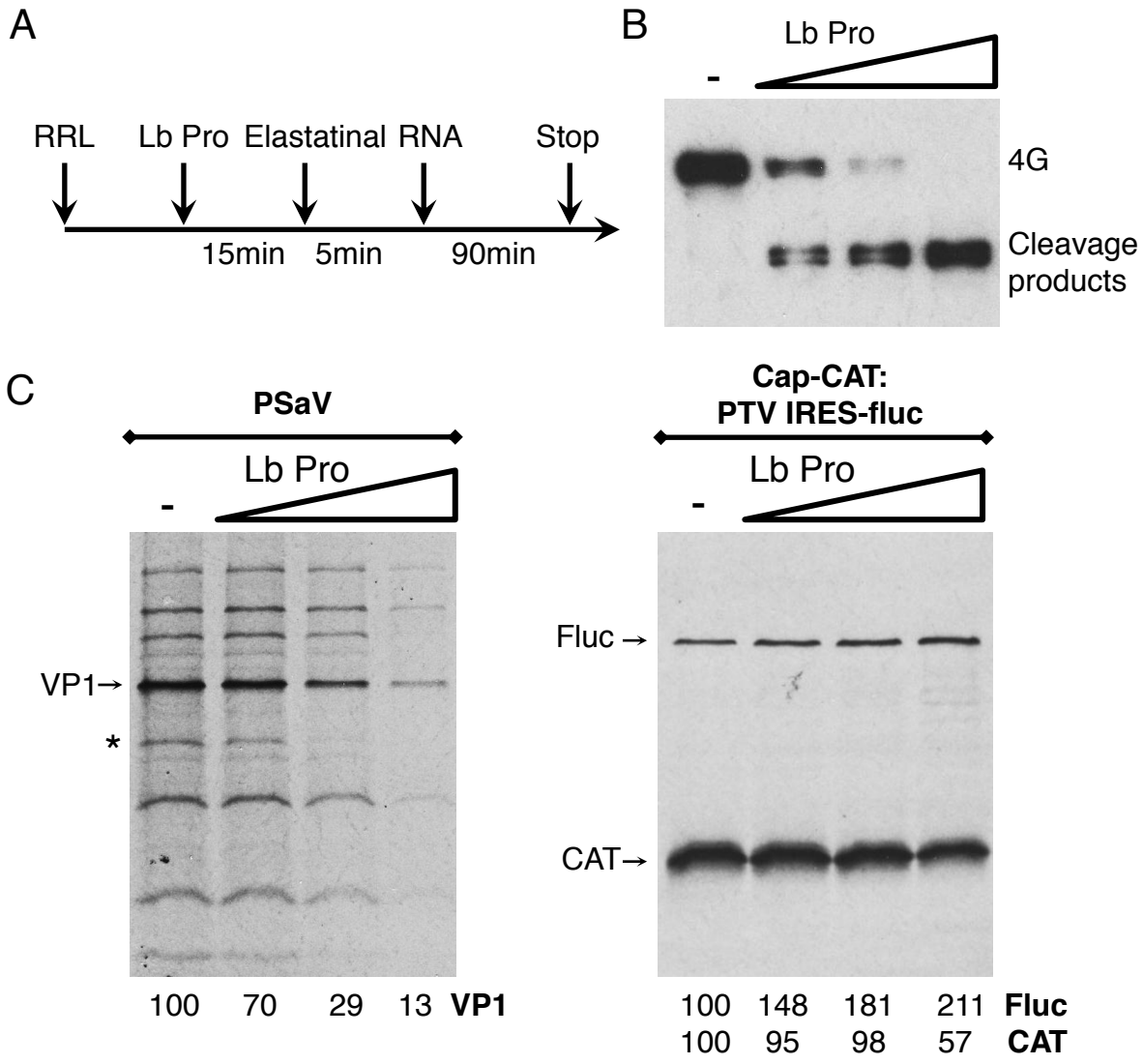
6 10 17 95 CAT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RRL
Lb Pro
Elastatinal
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Stop
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5min
90min
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Lb Pro
-
4G
Cleavage products
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PSaV
Lb Pro
-
VP1→
*
100
70
29
13
VP1
Cap-CAT:
PTV IRES-fluc
Lb Pro
-
Fluc →
CAT→
100
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100
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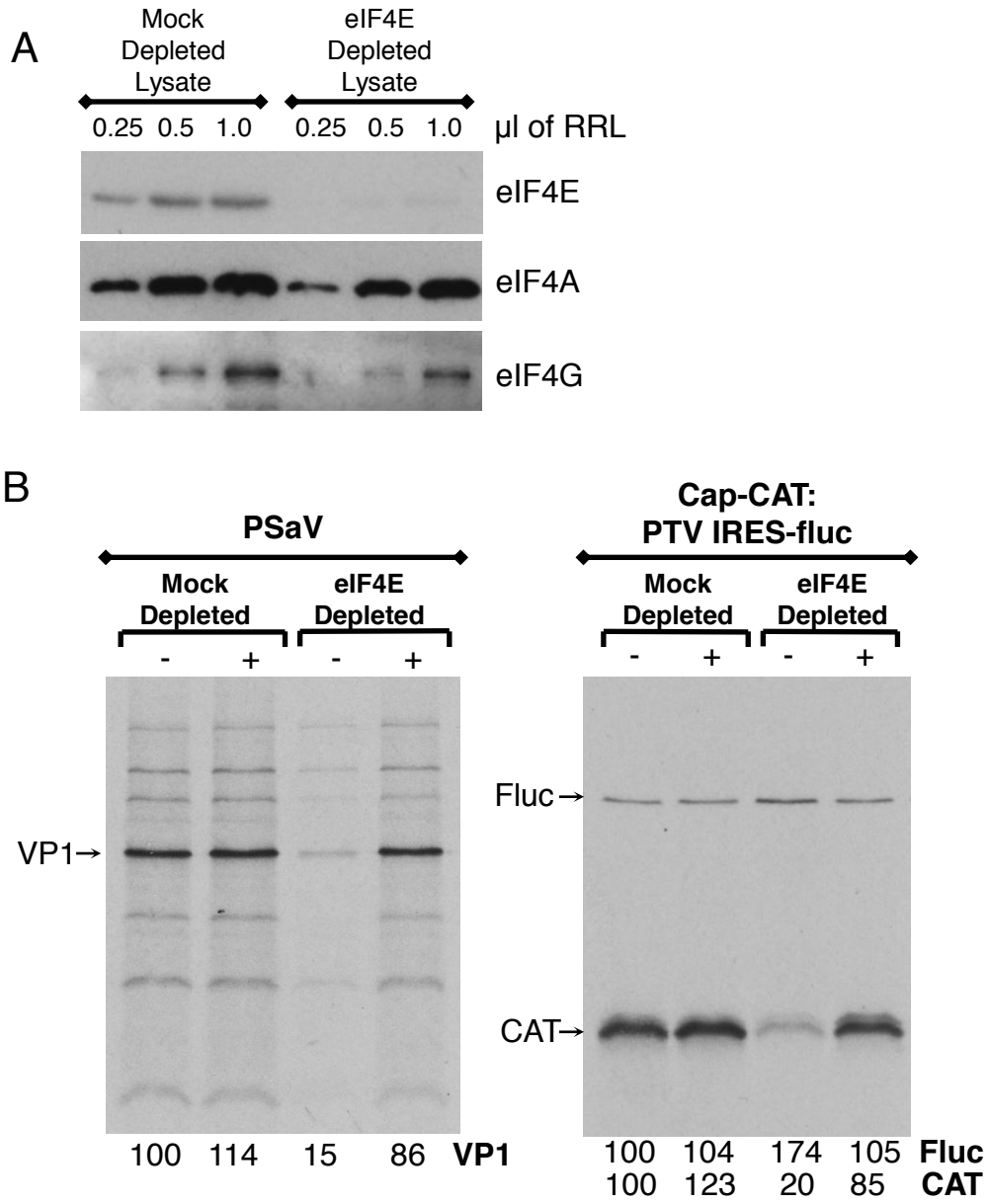
57
CAT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eIF4E Depleted Lysate
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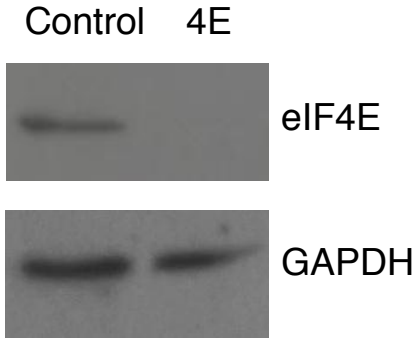
0.25 0.5 1.0
µl of RRL
eIF4E
eIF4A
eIF4G
B
PSaV
Cap-CAT: PTV IRES-fluc
Mock Depleted
eIF4E Depleted
- + - +
- + - +
VP1→
Fluc→
CAT→
100 114 15 86 VP1
100 104 174 105 Fluc
100 123 20 85 CAT

A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eIF4E
GAPDH

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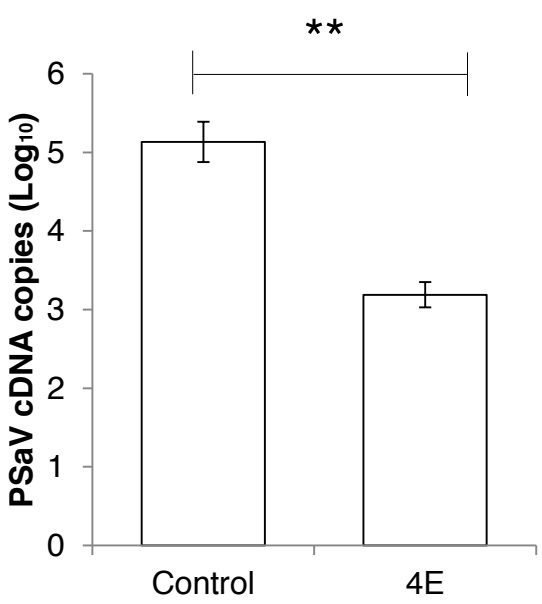
**
PSaV cDNA copies (Log10)
6
5
4
3
2
1
0
Control
4E