Rotavirus spike protein VP4 mediates viroplasm assembly by association to actin filaments.

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Abstract

The formation of viroplasms is a well-conserved step in the rotavirus (RV) life cycle. In these structures, both virus genome replication and progeny assembly take place. A stabilized microtubule cytoskeleton and lipid droplets are required for the viroplasm formation, which involves several virus proteins. The viral spike protein VP4 has not previously been shown to have a direct role in viroplasm formation. However, it is involved with virus-cell attachment, endocytic internalization, and virion morphogenesis. Moreover, VP4 interacts with actin cytoskeleton components, mainly in processes involving virus entrance and egress, and thereby may have an indirect role in viroplasm formation. In this study, we used reverse genetics to construct a recombinant RV, rRV/VP4-BAP, which contains a biotin acceptor peptide (BAP) in the K145-G150 loop of the VP4 lectin domain, permitting live monitoring. The recombinant virus was replication competent but showed a reduced fitness. We demonstrate that rRV/VP4-BAP infection, as opposed to rRV/wt infection, did not lead to a reorganized actin cytoskeleton as viroplasms formed were insensitive to drugs that depolymerize actin and inhibit myosin. Moreover, wt VP4, but not VP4-BAP, appeared to associate with actin filaments. Similarly, VP4 in co-expression with NSP5 and NSP2 induced a significant increase in the number of viroplasm-like structures. Interestingly, a small peptide mimicking loop K145-G150 rescued the phenotype of rRV/VP4-BAP by increasing its ability to form viroplasms and hence, improve virus progeny formation. Collectively, these results provide a direct link between VP4 and the actin cytoskeleton to catalyze viroplasm assembly.

IMPORTANCE The spike protein VP4 participates in diverse steps of the rotavirus (RV) life cycle, including virus-cell attachment, internalization, modulation of endocytosis, virion morphogenesis, and virus egress. Using reverse genetics, we constructed for the first time a recombinant RV, rRV/VP4-BAP, harboring a heterologous peptide in the lectin domain (loop K145-G150) of VP4. The rRV/VP4-BAP was replication-competent but with reduced fitness due to a defect in the ability to reorganize the actin cytoskeleton, which affected the efficiency of viroplasm assembly. This defect was rescued by adding a permeable small-peptide mimicking the wild-type VP4 loop K145-G150. In addition to revealing a new role of VP4, our findings suggest that rRV harboring an engineered VP4 could be used as a new dual vaccination platform providing immunity against RV and additional heterologous antigens.

Introduction

Rotavirus (RV) is the primary etiological agent responsible for severe gastroenteritis and dehydration in infants and young children worldwide (1), resulting in the death of 128'000 children under five years of age in developing countries. Moreover, it also infects young animals such as piglets, calves, and poultry, negatively impacting the livestock industry (2-4).

RV virions are non-enveloped icosahedral particles composed of three concentric layers, called triple-layered particles (TLPs). The virus core-shell encloses eleven double-stranded (ds) RNA genome segments and twelve copies of both the RNA-dependent RNA polymerase, VP1, and the guanyl-methyltransferase, VP3 (5, 6). The icosahedral core-shell (T=1, symmetry) is composed of twelve decamers of VP2 and surrounded by 260 trimers of the structural protein VP6, constituting transcriptionally active double-layered particles (DLPs) (7, 8). Trimers of VP7 glycoprotein organized in an icosahedral symmetry (T=13) stand on the top of each VP6 trimer constituting the main building component of the virion outer layer. The spike protein VP4 anchors at each of the virion 5-fold axes adopting a trimeric conformation although having a dimeric appearance when visualized from the top of the capsid surface (9-12).

Immediately after RV internalization, the DLPs are released into the cytoplasm and become transcriptionally active (13), leading to the first round of transcription, which is necessary for halting the host innate immune response (14-18), shutting off the host translation machinery (19), and starting the building-up of viroplasms (20-22). The RV cytosolic replication compartments, termed viroplasms, are membrane-less electron-dense globular inclusions responsible for virus genome replication and virus progeny assembly (20, 23). The RV proteins NSP5, NSP2, and VP2 are the assembling blocks for viroplasms

(24). Other virus proteins are also found in the viroplasms, including NSP4, VP1, VP3, and VP6, together with double-stranded and single-stranded viral RNAs. Host components, such as microtubules, lipid droplets, or miRNA-7 (25-27) are also recruited to viroplasms. The formation of viroplasms requires the reorganization and stabilization of the microtubule network and the recruitment of lipid droplets in a process directly associated with NSP2 and VP2. Thus, to be formed, the viroplasms have to coalesce from small-punctuated structures diffused in the cytosol at early times of infection to perinuclear large-mature viroplasms at late times of infection (25, 26, 28). However, pieces of evidence suggest that not only microtubules but also actin and intermediate filaments reorganize under RV infection by using a mechanism not yet described (25, 29). Many virus proteins have multifunctional roles during the viral life cycle, and the RV proteins are no exception. An example is VP4 which is cleaved by a trypsin-like enzyme found in the intestinal tract (30) into two main products, VP8* (28 kDa, amino acids 1-247) and VP5* (60 kDa, amino acids 248-776) that remain non-covalently associated with the infectious RV virion allowing the initialization of the virus entry process (31, 32). VP8* and a significant portion of VP5*, VP5CT (amino acids 246-477), constitute the distal globular and the central body of the spike, respectively (33, 34). The VP8* subunit has several functions, such as haemagglutinin activity (35), involvement in binding the host sialic acid (34), and a determinant role in virus tropism. VP5* has been implicated in the interaction with integrins (36-38). During virus internalization, VP4 engages the endocytic pathway (39, 40) by binding to the small GTPase Rab5 and PRA1 within early endosomes (41), directly activating hsp70 (42, 43) and associating with the actin-binding protein Drebrin 1, an RV restriction factor (44). VP4 also plays an essential role in virion morphogenesis and can be found as a soluble protein in the cytosol (45). When expressed in the absence of other RV proteins, VP4 is associated with

the microtubules and the actin cytoskeleton (45-50). In polarized cells, VP4 seems to interact with the apical actin cortex, leading to the remodeling of the brush border and subsequently releasing the RV virions into the medium (48). This actin association is dependent on a VP4 actin-binding domain present at its C-terminus (residues 713-776) in cooperation with the coiled-coil domain (residues 481-574) (45). However, there is no direct evidence that VP4 participates in actin reorganization during RV infection.

Here, we describe the generation of a recombinant RV (rRV) harboring a genetically modified genome segment 4 (gs4) encoding the spike protein VP4 with a biotin-acceptor peptide (BAP) tag of 15 amino acids inserted in an exposed loop of VP8* (residues K145-G150). Even though rRV/VP4-BAP is internalized and able to produce virus progeny, it has significantly reduced virus fitness because of an impaired ability of VP4-BAP to associate with the actin cytoskeleton. In addition, we provide clear evidence that VP4 acts as a catalyst for the assembly of the viroplasms in an actin-dependent process.

Results

Production and analysis of recombinant rotavirus harboring a BAP tagged spike protein. As the VP4 spike protein plays an essential role in the host cell tropism, attachment, and internalization, we addressed if VP4 could be engineered by incorporating a heterologous peptidic tag within its coding sequence without compromising its structural and functional properties. To test this hypothesis, we identified in the previously published crystal structure of simian RRV VP4 (10) four different exposed loops localized in the lectin domain (amino acids 65-224) of the VP8* subunit and then inserted a biotin acceptor peptide (BAP) tag (51, 52) in each of the corresponding loops of the VP4 simian strain SA11. As depicted in **Fig. 1a,** the selected loops for the BAP tag insertions were T96-R101, E109-

S114, N132-Q137, and K145-G150, labeled with the colors blue, orange, pink, and green, respectively. First, we assessed the biotinylation of these four BAP-tagged VP4 proteins, herein VP4-BAP, in cell lysates by western blot-retardation assay (WB-ra) (51). For this purpose, each construct was co-expressed with cytosolically localized biotin ligase Bir A (cyt-BirA) in MA104 cells infected with recombinant vaccinia virus encoding T_7 RNA polymerase to allow the synthesis of cytosolic VP4 transcripts. As the synthesis of nuclear VP4 transcripts promotes undesired mRNA splicing (53), we used a T_7 promoter to favor cytosolic transcription provided by the T_7 RNA polymerase. As shown in **Fig. 1b**, the four VP4-BAP variants (**Fig. 1b, lanes 4, 6, 8 and 10**), but not the wild type (wt) VP4 (**Fig. 1b, lane 2**), were fully biotinylated when the cell extracts were incubated with streptavidin (StAv), generating a band shift of approximately 140 kDa that corresponds to the VP4-BAP/StAv complex. Of note, biotinylated protein-StAV complexes hinder epitopes of the biotinylated protein denoted as a shifted and weaker band in western blot. The band observed immediately above the VP4-BAP band (80 kDa) represents a phosphorylated form of the protein that is only present in transfected cells but not in RV-infected cells, as demonstrated by the λ-phosphatase treatment of the cellular extracts (**Fig. S1a and b**). This result suggests that the expression and stability of the different VP4-BAP proteins were not affected by the location of the inserted BAP tag.

Next, we assessed whether the four VP4-BAP proteins could assemble into infectious RV particles and support virus replication. To rescue recombinant rotavirus (rRV) harboring a genetically modified genome segment 4 (gs4) encoding for the different VP4-BAP proteins (gs4-BAP), we took advantage of the previously established reverse genetics system (54). Of the four different constructs, we successfully rescued only the rRV harboring gs4-BAP encoding the BAP tag within the K145-G150 loop in VP8* (**Fig. 1a**),

herein named rRV/VP4-BAP. The rescued virus was confirmed by the differential migration pattern of the modified gs4-BAP compared to the wt gs4 (**Fig. 1c**) and by Sanger sequencing (**Fig. 1d**). Notably, the genome segment 4 (gs4-BAP) and all the other ten genome segments of rRV/VP4 were stable in tissue culture at least until virus passage ten as determined by Sanger sequencing and deep sequencing (**Fig S1b and supplementary information**). We then investigated the ability of rRV/VP4-BAP to express biotinylated VP4-BAP. Specifically, cell extracts of rRV/VP4-BAP infected MA/cytBirA cells (MA104 cells stably expressing cytosolic localized BirA) were analyzed at 6 hpi by western blot. Thus, the produced VP4-BAP protein showed to be biotinylated as demonstrated after incubation with StAv-peroxidase, which detected a band of approx. 85 kDa only in rRV/VP4-BAP infected MA/cytBirA cell extracts but not in rRV/VP4-BAP-infected MA104 cells (**Fig. 1e, lane 3 and 6**). As expected, VP4 biotinylation was detected neither in rRV/wt infected MA/cytBirA cells nor in rRV/VP4-BAP infected MA104 cells (**Fig. 1e, lanes 2, 5, and 6**). We found by WB-ra that the fraction of biotinylated VP4-BAP corresponds to 48 % of the total protein (**Fig. 1f**).

We next examined if the biotinylated virus-encoded VP4-BAP is incorporated into newly assembled virus particles. For this, we purified rRV/VP4-BAP virions produced in MA/cytBirA cells in the absence or presence of biotin and visualized the virus particles by negative staining electron microscopy after incubation with StAv conjugated to gold particles. Thus, 53% of the virions produced in the presence of biotin were decorated with StAV-gold particles (**Fig. 1g**) but none in the unbiotinylated control particles. Similarly, indirect ELISA with identical amounts of unbiotinylated and biotinylated purified rRV/VP4-BAP revealed a signal upon StAV-peroxidase staining only for biotinylated samples. Furthermore, similar signals were observed in ELISA using an anti-rotavirus antibody (**Fig.**

178 **1h**). These outcomes collectively suggest that virus-encoded VP4-BAP is biotinylated and
179 incorporated in newly formed RV particles. Interestingly, as shown in **Fig. 1i,** the rRV/VP4
180 particles appear to have a slightly larger diameter (~80 nm) when compared to rRV/wt
181 particles (~75 nm) but were still in the range of TLPs (55).

182 **Subcellular localization of rRV/VP4-BAP.** We investigated the subcellular localization of
183 the newly produced biotinylated VP4-BAP in rRV/VP4-BAP infected cells at 6 hpi, a time
184 point showing well-assembled viroplasms (25). For this, both rRV/wt and rRV/VP4-BAP-
185 infected MA/cytBirA cells were incubated in the absence or presence of biotin for 4 hours
186 before fixation. Biotinylated VP4-BAP detected with StAv-Alexa 555 was found
187 surrounding the viroplasms (stained anti-NSP5) (**Fig. 2a**). However, no StAv-Alexa 555
188 signal was detected in cells infected with either rRV/wt or rRV/VP4-BAP without biotin.
189 Notably, the biotinylated VP4-BAP partially co-localized with trimeric VP7 in the
190 endoplasmic reticulum (ER) (**Fig. 2b**). Additionally, the cytosolic distribution of VP4-BAP
191 was similar to that of VP4 in rRV/wt-infect cells **(Fig. 2c)**. These results suggest that the
192 modification exerted in VP4-BAP does not impact VP4 subcellular localization in infected
193 cells.

194 **Impaired virus fitness of rRV/VP4-BAP**. To compare the replication fitness of rRV/VP4-
195 BAP with that of rRV/wt, we infected MA104 cells at equal MOI and harvested the virus at
196 various time points until 48 hpi. As depicted in **Fig 3a**, rRV/VP4-BAP showed a significantly
197 delayed fitness curve compared to rRV/wt. To investigate this divergence in the virus
198 replication, we infected cells with identical MOI (**Fig. 3b**) or an equal number of virus
199 particles (**Fig. 3c**) and quantified cells showing viroplasms at 6 hpi. In both experimental
200 conditions, we observed a significantly reduced ratio of cells containing viroplasms upon
201 rRV/VP4-BAP infection when compared with rRV/wt infection.

Comparable entry processes between rRV/wt and rRV/VP4-BAP. Since VP4 has an essential role in virus-cell attachment, we interrogated whether the ability of rRV/VP4-BAP particles to bind to cells was impaired. To test this hypothesis, we performed a nonradioactive binding assay described previously by Zarate *et al.* (56) to compare the attachment to MA104 cells with different amounts of either rRV/wt or rRV/VP4-BAP. As depicted in **Fig 4a**, no differences in cell attachment were observed among the two viruses. Of note, the antibody conditions used for the virus detection are in the linear range (**Fig S2a**). Moreover, biotin-labeled rRV/VP4 did not hinder the virus-cell attachment as denoted by the same results for the attachment of both unbiotinylated and biotinylated rRV/VP4-BAP virus particles (**Fig 4b**). As expected, binding of rRV/VP4-BAP was detected with StAVonly when grown in the presence of biotin (**Fig 4c**).

Next, we investigated whether the delay in viral replication fitness was caused by a difference in virus internalization. Purified rRV/VP4-BAP and rRV/wt virions were compared and analyzed for virus internalization by confocal scanning laser microscopy (CSLM) using immunostaining with the conformational monoclonal antibody anti-VP7 (clone 159), which only recognizes the trimeric form of the VP7 protein (57, 58). As a control, purified rRV/VP4-BAP, but not rRV/wt, was directly labeled with StrAv-Alexa 555 prior to infection. Initially (0 min), VP4-BAP and VP7 signals co-localized on the cell surface, indicating the association of virions to the cell membrane. However, after two minutes at 37°C, both signals were already internalized (**Fig 4d**). The localization patterns were comparable to those observed at the same time points with rRV/wt virions, suggesting no differences in the internalization mechanism between the two viruses.

Since virus-cell attachment and virus internalization were comparable between rRV/wt and rRV/VP4, we investigated if virus transcription was defective or delayed for

rRV/VP4-BAP. Thus, we compared the abundance of NSP5 and VP6 virus transcripts at 4 hpi of MA104 cells infected with either rRV/wt or rRV/VP4-BAP. As denoted in **Figs 4e-g**, the transcription levels of NSP5, VP6, and the housekeeping gene SDHA were comparable in cells infected with rRV/wt or rRV/VP4-BAP.

rRV/VP4-BAP has a defect in a step between virus transcription and viroplasm formation. We analyzed by high-definition electron microscopy the structural morphology of the viroplasms from rRV/wt or rRV/VP4-BAP at two time points, 6 and 12 hpi, which for simian strain SA11 corresponds to a time showing well-formed viroplasms and a time with highly mature viroplasms, respectively (**Fig 5a**). No apparent differences in the viroplasm morphology were observed between the two viruses at 6 and 12 hpi. Similarly, we examined whether the liquid-liquid phase separation properties of rRV/VP4-BAP viroplasms were modified. For this, we took advantage of our previously established MA104 cell line stably expressing NSP2-mCherry (MA/NSP2-mCherry) to visualize viroplasm formation in living cells because of the ability of this protein to get recruited into viroplasms during RV infection (21, 25, 59). Next, we measured the NSP2-mCherry diffusion dynamics in single viroplasms using fluorescence recovery after photobleaching (FRAP) experiments (**Fig 5b and c**). We found that FRAP properties concerning NSP2-mCherry half-time recovery and mobility were similar for both viruses (**Fig. 5d and e**).

To confirm our results and exclude the involvement of the endocytic pathway, we purified and transfected MA104 cells with an equal number of DLPs of the two viruses (**Fig 6a**). Of note, purified rRV/wt DLPs or rRV/VP4-BAP DLPs had the same size, while rRV/VP4-BAP TLPs were larger than rRV/wt TLPs (**Fig 1i and 6a**). At 6 h after transfection, we observed identical expression of NSP5 for both viruses, as denoted by in-cell western assay **(Figs 6b and c)**. In the same experimental setting, the number of cells showing

viroplasms (detected with anti-NSP5) was significantly reduced in rRV/VP4-BAP infected cells compared to rRV/wt infected cells (**Figs 6d and e**). These outcomes strongly suggest that the reduced replication fitness of rRV/VP4-BAP involves a step in viroplasm assembly.

VP4 promotes viroplasm assembly. Since we hypothesized that VP4 may have a yet unidentified role in the viroplasm assembly, we investigated if spontaneous disruption of the viroplasm led to a delayed re-assembly of these structures. To challenge this hypothesis, we used 1,6-hexanediol (1,6-HD), a well-described aliphatic alcohol able to disrupt key drivers of liquid-liquid phase separation and recently shown to be effective in dissolving RV viroplasms (28) and determined the recovery of NSP2-mCherry in viroplasms. For this, MA/NSP2-mCherry cells at 5 h after infection with either rRV/VP4-BAP or rRV/wt were treated for 6 min with 1,6-HD and observed for 30 min after washout of the compound (**Fig. 7a and b**). Interestingly, the recovery kinetic of rRV/VP4-BAP viroplasms was delayed compared to that of rRV/wt at short times after removing the drug (2 min), as confirmed by both a reduced ratio of cells presenting viroplasms (**Fig. 7c**) and a reduced number of viroplasm per cell (**Fig. 7d**). In order to further characterize the relationship between viroplasms and VP4, we performed transcriptional silencing experiments with VP4 specific siRNAs (siVP4) in MA/NSP2-mCherry cells (**Fig. 7e**). In this context, it has been previously described that the silencing of VP4 during RV infection leads to viroplasm formation even if TLPs assembly is impaired (60). We then monitored viroplasm formation in siVP4 and control-siRNA (scr) transfected MA104 cells infected with simian strain SA11 and treated with 1,6-HD as described for the experiment shown in **Fig 7a**. The viroplasms produced on VP4 silenced cells had a delayed recovery kinetic similarly to that observed for rRV/VP4-BAP viroplasms (**Fig 7f and g**). Furthermore, similarly to **Fig 7d,** the number of viroplasms per cell was significantly decreased in siVP4 treated cells at 2 min of recovery

274 compared to the experimental controls (**Fig 7h**). Thus, these results strongly suggest that
275 VP_4 promotes either indirectly or directly the assembly of viroplasms.
276 **VP_4-BAP impairs VLS assembly and association to actin filaments.** Viroplasm-like
277 structures (VLS) are simplified models for the study of complex viroplasm structures,
278 requiring the co-expression of NSP_5 with either NSP2 (24) or VP2 (22) to form globular
279 cytosolic inclusions morphologically similar to viroplasms but unable to replicate and
280 produce virus progeny. Considering this rationale, we formed VLSs by expressing NSP_5 and
281 NSP2 in the presence of GFP, VP_4-GFP, or VP_4-BAP-GFP (**Fig 8a**). We noticed the absence
282 of co-localization of either VP_4-GFP or VP_4-BAP-GFP in VLSs. Furthermore, when we
283 quantified the number of VLSs per cell (**Fig 8b**), we noticed that the number of VLSs
284 formed in the presence of VP_4-GFP was much larger than that produced in the presence of
285 VP_4-BAP-GFP or GFP.
286 Since VP_4 associates with actin cytoskeleton components (45, 47, 48), we
287 hypothesized that VP_4-BAP-GFP could have an impaired association with actin filaments.
288 To investigate this possibility, MA104 cells expressing VP_4-GFP or VP_4-BAP-GFP were
289 untreated or treated with nocodazole for 1h before fixation. Nocodazole treatment induces
290 depolymerization of the microtubule network permitting direct characterization of proteins
291 associated with the actin cytoskeleton. In this context (**Fig 8c**), we stained the cells to
292 detect actin cytoskeleton and noticed that VP_4-GFP was associated, as expected, with
293 actin filaments even after nocodazole treatment; while VP_4-BAP-GFP was associated with
294 filaments only in the absence of nocodazole. In MT-depolymerized cells, VP_4-BAP-GFP
295 formed diffuse small cytosolic aggregates or short filaments. Moreover, while the length of
296 VP_4-GFP fibers was comparable in the presence or absence of nocodazole treatment, the
297 VP_4-BAP-GFP fibers were significantly shorter in cells treated with nocodazole (**Fig 8d**),

298 suggesting an impaired association of VP4-BAP with actin filaments. Taken together, these
299 results suggest that VP4 promotes the assembly of VLSs mediated by its association with
300 actin filaments.

301 **Impaired association between the actin cytoskeleton and rRV/VP4-BAP results in**
302 **delayed viroplasm assembly.** RV strain SA11 infection (**Fig 9a** and (25, 61)) reorganizes the
303 actin cytoskeleton, mainly by decreasing the actin stress fiber and redistributing it to the
304 cell cortex. However, this reorganization did not take place in cells infected with rRV/VP4-
305 BAP (**Fig 9b and Fig S3a**) featured by an increment in stress fibers surrounding the
306 viroplasms (**Fig 9b**, yellow open arrows) and contrasting by an actin cell cortex increment in
307 rRV/wt infected cells. Moreover, the actin cytoskeleton is not properly reorganized even in
308 experiments with an increased rRV/VP4-BAP multiplicity of infection (**Fig. S3b**).
309 Interestingly, the MT-network reorganization was attained by both viruses.

310 The cell has a contractile system regulated in part by the reorganization of stress
311 fibers composed by actin and myosin II. We questioned if the molecular motor myosin was
312 also required for viroplasm assembly and if its activity was impaired in rRV/VP4-BAP
313 infected cells. For this purpose, we inspected the localization of paralog myosin IIa in cells
314 infected with either rRV/wt or rRV/VP4-BAP at 6 hpi and compared it with non-infected
315 conditions (**Fig 9c**). In non-infected cells, myosin IIa is homogenously distributed in
316 filaments, stacks, clusters, and continuous structures (62). However, upon rRV/wt infection,
317 continuous myosin structures are lost, and myosin clusters and stacks in the cell cortex are
318 favored (**Fig 9c,** yellow open arrowheads). In contrast, rRV/VP4-BAP infected cells still have
319 continuous structures, mainly in the ventral cell region. Interestingly, VP4 fibrillar
320 morphology is observed in rRV/wt infected cells (**Fig 9b and c**, red open arrows) but not in
321 rRV/VP4-BAP infected cells.

We confirmed the active role of the actin cytoskeleton in the assembly of rRV/wt and rRV/VP4-BAP viroplasms by adding actin inhibitors jasplakinolide (jasp) and cytochalasin B (cyt B) at 1 hpi, a time in which virus internalization and primary virus transcription are well-initiated (**Fig 9d**). In this context, we denoted that rRV/wt viroplasms were sensitive to jasp and cyt B treatment, as the ratio of cells showing viroplasms (**Fig 9e**) and the number of viroplasms per cell (**Fig 9f**) were significantly decreased, reaching the levels observed in rRV/VP4-BAP infected cells in presence or absence of actin inhibitors.

We next inhibited the non-muscular myosin II using blebbistatin (BLB)(63), a small molecule inhibiting both myosin II paralogs a and b (**Fig 9g-i**). Like the actin inhibitors, blebbistatin reduced the ratio of cells with viroplasms (**Fig 9h**) and the number of viroplasms per cell (**Fig 9i**) in cells infected with rRV/wt but not with rRV/VP4-BAP. Our results suggest that viroplasm assembly requires actin and myosin II in a mechanism employing VP4.

A small peptide mimicking loop K145-G150 rescues the rRV/VP4-BAP phenotype. It has been described that the C-terminal region of VP5* harbors an actin-binding domain (45). We hypothesize that the insertion of a BAP tag in loop K145-G150 interferes with the association of VP5* with the actin cytoskeleton, which results in a delay in viroplasm assembly because of an inability to reorganize the actin cytoskeleton. To prove this hypothesis, we designed a small peptide harboring the amino acid sequence of wt VP4 loop K145-G150 flanked at the N-terminus by an arginine-tail and at the C-terminus by conjugation to fluorescein isothiocyanate (FITC), for peptidic internalization and visualization, respectively. Thus, the sequence of this small peptide corresponds to RRRRRR^{143}VV^{145}KTTANG^{150}SIGQYG^{156}-FITC, and was designated small peptide loop K145-G150 (SPL). Of note, SPL was not toxic for cells up to a concentration of 100 µM even after

24 h of treatment (**Fig S4a**). SPL was internalized in cells at 2 h post-treatment and found diffuse in the cytosol **(Fig S4b)**. In the first instance (**Fig 10a**), we expressed VP_4-GFP and VP_4-BAP-GFP (stained with anti-GFP, red) in BHK-T_7 cells in the absence or presence of SPL and monitored the filamentous distribution of these proteins. Interestingly, as quantified in **Fig 10b**, the addition of SPL increased the length of VP_4-BAP-GFP filaments compared to the untreated sample reaching the same level of the fiber lengths as VP_4-GFP. The filamentous distribution of VP_4-GFP did not change with SPL treatment. We next interrogated if SPL can improve the replication fitness of rRV/VP_4-BAP. For this, SPL was added at 1 or 3 hpi to MA104 cells infected with either rRV/wt or rRV/VP_4-BAP and then monitored at 6 hpi for viroplasm formation (**Fig 10c**). Surprisingly, the addition of SPL at 1 hpi improved drastically the number of cells presenting viroplasms in rRV/VP_4-BAP-infected cells, reaching similar levels as observed in rRV/wt infected cells (**Fig 10d**). Consistently, the viroplasm size and numbers per cell increased upon SPL treatment at 1 hpi (**Fig 10e and f**). The addition of SPL at 3 hpi resulted in increased viroplasm size but not increased numbers per cell. The treatment of rRV/wt infected cells with SPL did not affect viroplasm size and numbers (**Fig S5c-d**). We then tested the ability of SPL to recover rRV/VP_4-BAP virus progeny. For this purpose, rRV/VP_4-BAP infected cells were treated at 1 hpi with SPL, and virus progeny was recovered at 12 hpi. As denoted in **Fig 10g**, rRV/VP_4-BAP virus progeny formation significantly increased upon treatment with SPL, while the same treatment had no effect on rRV/wt progeny formation. We concluded that SPL boosted the association of VP_4-BAP to actin-bundles, thereby rescuing the assembly of viroplasms with a concomitant increase in the production of virus progeny.

Discussion

The external coat layer of the RV virion can be modified *in vitro* by adding a specific ratio of VP7 and VP4 proteins to purified DLPs to generate recoated TLPs (rcTLPs) (10-12, 64). This approach provides a valuable tool for studying the VP4 structural requirements for virion internalization (10). However, rcTLPs have methodological limitations and do not allow transferring the parental phenotype to the virus progeny. Moreover, rcTLPs only allow single amino acid substitutions in the VP4 spike protein (65, 66). However, the recently implemented entirely plasmid-based RV reverse genetics technology resulted in a valuable tool for studying several aspects of RV replication and the development of future RV based vaccines because it supports site-directed modifications of RV genome segments of interest. Specifically, RV reverse genetics technology has demonstrated that gs4, encoding VP4, can be artificially reassorted or recombined by transferring human RV strain gs4 into a simian strain SA11 backbone (54).

In the present study, we used an RV reverse genetics system to remodel the spike protein VP4 by incorporating a BAP tag in four independent exposed loops in the VP8* lectin domain. Interestingly, although the four differently modified proteins were efficiently biotinylated in transfected cells, only one of them harboring a BAP tag inserted in the K145-G150 loop, rRV/VP4-BAP, was rescued using RV reverse genetics (54). We hypothesize that those VP4-BAP versions, which were unable to be rescued, could have had strongly compromised their virus progeny because they directly impacted, and as denoted by *in silico* modeling of these structures (**Fig S5a**), *i)* the VP4 transition states from upright (immature) to reverse (mature) (65), *ii)* the VP4 association with specific cellular receptors (36, 37), or *iii)* the incorporation of VP4 in the coat layer.

VP4-BAP and VP4 share similar distribution patterns in infected cells, such as their localization surrounding viroplasms, co-localization in the endoplasmic reticulum with VP7, and incorporation into newly assembled virions. However, we found that rRV/VP4-BAP had a significantly reduced replication fitness compared to rRV/wt. To identify the reason for this, we investigated the various steps involved in RV replication. We show that rRV/VP4-BAP internalization kinetics are comparable to those of the rRV/wt. This internalization kinetics consists of a sequence of events starting with the interaction of the virus with the cell membrane and concluding with the decrease of calcium levels within the endosomes, provoking the loss of the outer layer of the virion, which triggers the release of transcriptionally active DLPs into the cytosol (66-69). We also confirmed that virus transcription and translation are comparable between both viruses. Moreover, there were no differences in rRV/VP4-BAP viroplasm morphology and liquid-like dynamics, as determined by high-resolution electron microscopy and NSP2-mCherry mobility on single-viroplasm FRAP experiments, respectively. However, upon transfection with rRV/VP4-BAP DLPs that bypass the virus physiological internalization pathway, showed a delayed viroplasm formation compared to rRV/wt DLPs, even though NSP5 expression levels were equivalent for both viruses. Collectively, these results indicate that the reduced replication fitness of rRV/VP4-BAP is due to a step between primary virus translation and viroplasm formation. We hypothesize that rRV/VP4-BAP, and specifically VP4-BAP, was defective in associating with the actin cytoskeleton because we observed *i)* a delayed recovery of rRV/VP4-BAP viroplasms after 1,6-HD treatment, *ii)* inability of VP4-BAP-GFP to increase the VLS counts, *iii)* absence of co-localization of VP4-BAP-GFP with actin stress fibers, *iv)* inability of rRV/VP4-BAP to reorganize actin cytoskeleton, and *v)* insensitivity of rRV/VP4-BAP viroplasms for drugs depolymerizing actin filaments and inhibiting myosin II molecular

416 motor. In contrast, rRV/wt infection led to the reorganization of the actin cytoskeleton, and
417 rRV/wt viroplasms were sensitive to actin-depolymerizing and myosin inhibitor drugs
418 linking actin with viroplasm assembly. Additionally, the numbers of VLS duplicated when
419 co-expressed with VP4-GFP. Previous studies (45, 49) demonstrated the ability of VP4 to
420 associate with actin in the absence of other virus proteins, mainly through an actin-binding
421 domain present in the C-terminal region of VP5* (residues 713-776) when in cooperation
422 with the coiled-coil domain (residues 481-574). In this context, we also present evidence
423 that the sole expression of VP4 allows its association with actin. But importantly, by
424 employing rRV/VP4-BAP infection, we show that the association of VP4 with actin is
425 essential to catalyze the formation of viroplasms. This finding provides an additional
426 function to VP4 in RV replication.

427 Interestingly, incorporating a small peptide mimicking loop K145-G150 of VP4
428 during rRV/VP4-BAP infection reverted the impaired formation of viroplasm with a
429 significant improvement in virus progeny production. We concluded that rRV/VP4-BAP
430 replication is enhanced because SPL raised the association of actin with VP4-BAP during
431 virus infection and because we observed that VP4-BAP, in the absence of other viral
432 proteins, associated with actin filament when expressed with SPL. We, therefore,
433 hypothesize that the VP8* subunit is involved in at least one of these three aspects that
434 render the viroplasms assembled and stabilized: *i)* association of VP8* with a yet
435 undescribed host component, *ii)* a reorganization of VP5*-VP8* association or *iii)* a direct
436 role of VP8* over another RV protein in viroplasm assembly. The role of the actin
437 cytoskeleton in the RV life cycle, particularly internalization and egress, has been previously
438 demonstrated (39, 47, 70). Here, we show that the actin cytoskeleton is also involved in
439 viroplasm formation, an essential intermediate stage of RV replication. It has been

440 described that the silencing of Rac1, a member of the Rho family of small GTPases playing
441 a major role in actin- and microtubule-cytoskeleton dynamics, leads to a decrease in RV
442 progeny formation in a process downstream of cell entry (70). We therefore cannot exclude
443 activation of Rac1 by VP4, allowing reorganization of the actin cytoskeleton for an efficient
444 assembly of the viroplasms.

445 Finally, specific *in vivo* biotinylation of cellular targets can be achieved by adding a
446 BAP tag to the protein of interest and co-expressed with the *E.coli*-derived biotin ligase,
447 BirA (71). This method is a powerful tool for versatile applications, such as identifying highly
448 complex interactomes (72, 73), and permits batch protein and subviral particle (51, 52)
449 refinement at high purity and in physiological conditions. The incorporation of a BAP tag in
450 RV VP6, allowed the preparation and purification of replication-competent DLPs (52).
451 Identifying a permissive target site in loop K145-G150 of VP4 spike protein for the insertion
452 of an exogenous peptide may impact the RV field. This VP4 modification favors the
453 insertion of peptides required for super-resolution microscopy or DNA-paint technologies
454 (e.g., Halo or BC2 tags) to dissect debated aspects of RV entry. In addition, this VP4
455 modification technology could permit the incorporation of antigenic peptides for vaccine
456 development. Although it is well-known that the current oral RV vaccines elicit an efficient
457 immune response (74, 75), rRV harboring a modified VP4 could provide an improved
458 vaccination platform for the display of other antigens fostering the development of a new
459 generation of dual vaccines.

460

461 **Material and Methods**

462 **Cells and viruses.** MA104 cells (embryonic African green monkey kidney cells; ATCC CRL-
463 2378) were grown in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies)

containing 10% fetal calf serum (FCS) (AMIMED; BioConcept, Switzerland) and penicillin (100 U/ml)–streptomycin (100 µg/ml) (Gibco, Life Technologies). MA/cytBirA and MA/NSP2-mCherry (59) cell lines were grown in DMEM supplemented with 10% FCS, penicillin (100 U/ml)-streptomycin (100µg/ml) and 5µg/ml puromycin (InvivoGen, France). BHK-$T_{7/9}$ (baby hamster kidney stably expressing T_7 RNA polymerase) cells were kindly provided by Naoto Ito (Gifu University, Japan)(76) and cultured in Glasgow medium supplemented with 5% FCS, 10% tryptose phosphate broth (Sigma-Aldrich), 10% FCS, penicillin(100 U/ml)-streptomycin (100µg/ml), 2% nonessential amino acids and 1% glutamine.

rRV/wt (59), rRV/VP4-BAP, and simian rotavirus strain SA11 (G3P6[1])(77) were propagated, grown, and purified as previously described (78). Virus titer was determined as viroplasm forming units per ml (VFU/ml) as described by Eichwald et al. 2012 (25). The T_7 RNA polymerase recombinant vaccinia virus (strain vvT7.3) was amplified as previously described (79).

Cell line generation. MA/cyt-BirA cell line was generated using the PiggyBac technology (80). Briefly, $1x10^5$ MA104 cells were transfected with the pCMV-HyPBase (80) and transposon plasmids pPB-cytBirA using a ratio of 1:2.5 with Lipofectamine™ 3000 transfection reagent (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's instructions. The cells were maintained in DMEM supplemented with 10% FCS for three days and then selected for four days in DMEM supplemented with 10% FCS and 5 µg/ml puromycin (59).

Reverse genetics. rRV/VP4-BAP was prepared as described previously (59, 81) using a pT_7-VP4-BAP instead of pT_7-VP4. Briefly, monolayers of BHK-T_7 cells ($4×10^5$) cultured in 12-well plates were co-transfected using 2.5 µl of TransIT-LT1 transfection reagent (Mirus) per

488 microgram of DNA plasmid. The mixture comprised 0.8 µg of SA11 rescue plasmids: pT_7-
489 VP1, pT_7-VP2, pT_7-VP3, pT_7-VP4-BAP, pT_7-VP6, pT_7-VP7, pT_7-NSP1, pT_7-NSP3, pT_7-NSP4,
490 and 2.4 µg of pT_7-NSP2 and pT_7-NSP5 (82, 83). Additionally, 0.8 µg of pcDNA3-NSP2 and
491 0.8 µg of pcDNA3-NSP5, encoding NSP2 and NSP5 proteins, were co-transfected to
492 increase rescue efficiency (59, 81). Next, cells were co-cultured with MA104 cells for three
493 days in serum-free DMEM supplemented with trypsin from porcine pancreas (0.5 µg/ml
494 final concentration) (T0303-Sigma Aldrich) and lysed by freeze-thawing. Then, 300 µl of the
495 lysate was transferred to new MA104 cells and cultured at 37°C for four days in serum-free
496 DMEM supplemented with 0.5 µg/ml trypsin until a visible cytopathic effect was observed.
497 The modified genome segments of rescued recombinant rotaviruses were confirmed by
498 specific PCR segment amplification followed by sequencing (59).
499 **Antibodies and Chemicals.** Guinea pig anti-NSP5, guinea pig anti-RV, goat anti-RV, mouse
500 monoclonal anti-NSP5 (clone 2D2), and rabbit anti-VP4 were described previously (25, 84-
501 87). Mouse monoclonal anti-VP5 (clone 2G4) and mouse monoclonal anti-VP7 (clone 159)
502 was kindly provided by Harry Greenberg (Stanford University, CA, USA). Rabbit anti-simian
503 rotavirus VP4 was purchased from Abcam. Mouse mAb anti-glyceraldehyde
504 dehydrogenase (GAPDH) (clone GAPDH-71.1), mouse anti-alpha tubulin (clone B-5-1-12),
505 and mouse mAb anti-β-actin (clone AC-74) were purchased to Merck. Mouse mAb anti-GFP
506 clone C-2) was purchased from Santa Cruz Biotechnology, Inc. Mouse mAb anti-α-tubulin
507 was directly conjugated to Atto 488 using lightning-link™Atto 488 conjugation kit from
508 Innova Bioscience, UK. Streptavidin-HRP was purchased from Merck. Streptavidin-Alexa
509 555 and secondary antibodies conjugated to Alexa 488, Alexa 594, Alexa 647, Alexa 700
510 (ThermoFisher Scientific).

511 1,6-hexanediol, nocodazole, jasplakinolide, and cytochalasin B were purchased from
512 Merck. (-)-Blesbbistatin was purchased from Cayman Chemical, USA.
513 Amino acids and their derivatives were purchased from Advanced ChemTech,
514 Novabiochem, Iris Biotech GMBH, Sigma-Aldrich, PolyPeptide, Space peptides and GL
515 BioChem. Amino acids were used as the following derivatives Fmoc-Arg(Pbf)-OH, Fmoc-
516 Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Lys(Fmoc)-OH, Fmoc-Pro-OH, Fmoc-Val-OH, Fmoc-
517 Trp(Boc)-OH, Fmoc-Ile-OH, Fmoc-Gln(Trt)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Ala-OH and Rink
518 Amide AM resin (loading: 0.38 $mmol \cdot g^{-1}$) and were purchased from Sigma Aldrich.
519 OxymaPure (hydroxyiminocyanoacetic acid ethyl ester) and DIC (*N*,*N*′-diisopropyl
520 carbodiimide) were purchased from Iris Biotech GMBH. 5(6)-carboxyfluorescein (CF) was
521 from Sigma. EM104 10ml glass syringes from Sanitex international.
522
523 **DNA plasmids.** pcDNA-VP4-SA11 was obtained by RT-PCR amplification of VP4 ORF of
524 gs4 of simian rotavirus strain SA11 (88) using specific primers to insert *Hind*III and *Xho*I
525 sites, followed by ligation into those sites in pcDNA3 (Invitrogen). pcDNA-VP4-*Kpn*I/*BamH*I
526 was built by insertion of point mutations in pcDNA-VP4-SA11 using the QuikChange site-
527 directed mutagenesis kit and protocol (Agilent) to insert *Kpn*I and *BamH*I restriction sites in
528 VP4. pcDNA-VP4-BAP (blue), (orange), (pink), and (green) were obtained by ligation
529 between *Kpn*I and *BamH*I of pcDNA-VP4-*Kpn*I/*BamH*I, a synthetic DNA fragment
530 (GenScript®) containing BAP tag in VP4 loops in amino acid regions 96-101, 109-114, 132-
531 137 and 145-150, respectively. The BAP tags are flanked by *BspE*I and *Nhe*I restriction sites
532 for easy tag replacement.
533 RV plasmids pT_7-VP1-SA11, pT_7-VP2-SA11, pT_7-VP3-SA11, pT_7-VP4-SA11, pT_7-VP6-
534 SA11, pT_7-VP7-SA11, pT_7-NSP1-SA11, pT_7-NSP2-SA11, pT_7-NSP3-SA11, pT_7-NSP4-SA11,

and pT_7-NSP5-SA11 were previously described (82). pcDNA3-NSP5 and pcDNA3-NSP2 were already described (59). pT_7-VP4-BAP (blue), (orange), (green), and (pink) were obtained by inserting a synthetic DNA fragment (Genscript) encoding for the VP4 protein-encoding BAP tag flanked by *Mfe*I and *Nde*I restriction enzymes sites and ligated into those sites in the pT_7-VP4-SA11.

pPB-cytBirA was obtained from a synthetic DNA fragment (Genscript) containing the BirA enzyme open reading frame of *Escherichia coli* (UniProt accession number: P06709) and inserted in the pPB-MCS vector (81) using *Nhe*I-*BamH*I restriction enzymes sites.

pcDNA-NSP5(SA11) and pcDNA-NSP2(SA11) were previously described (24, 89). pCI-VP4-GFP and pCI-VP4-BAP plasmids were obtained from PCR amplification of pT_7-VP4-SA11 and pT_7-VP4-BAP (green) using specific primers to insert *Nhe*I and *Mlu*I sites, followed by ligation in-frame on those sites in pCI-GFP. Thus, the GFP fragment was PCR amplified from pEGFP-N1 (Clontech) using specific primers to insert *Mlu*I/*Not*I restriction enzyme sites and ligated on those restriction enzyme sites into pCI-Neo (Promega). All the oligonucleotides were obtained from Microsynth AG, Switzerland. A list of all DNA sequences synthesized is provided in **Table S1**.

Streptavidin-supershift assay and immunoblotting. The assay was performed as described by Predonzani et al (51). Briefly, cell extracts were lysed in TNN lysis buffer (100mM Tri-HCl pH8.0, 250 mM NaCl, 0.5% NP-40, and cOmplete protease inhibitor (Roche)) and centrifuged for 7 min at 15'000 rpm and 4˚C. The supernatant was exhaustively dialyzed against PBS (phosphate-buffered saline, 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4 pH 7.2) at 4˚C and heated for 5 min at 95˚C in Laemmli sample buffer. Samples were incubated for 1 h at 4˚C with 1 µg streptavidin (Sigma) and

559 then resolved in SDS-polyacrylamide gel under reducing conditions. Proteins were
560 transferred to nitrocellulose 0.45 µm (90) and incubated with corresponding primary and
561 secondary antibodies. Secondary antibodies were conjugated to IRDye680RD or
562 IRDye800RD (LI-COR, Germany) for protein detection and quantification in Odyssey® Fc
563 (LI-COR Biosciences).

564 **Virus fitness curve.** The experiment was performed as described previously (91) with some
565 modifications. MA104 cells (1×10^5) seeded in 24-well plates were infected with rRV at an
566 MOI of 10 VFU/cell. The virus was allowed to adsorb for 1 h at 4°C, followed by incubation
567 at 37°C in 500 µl DMEM. At the indicated time points, the plates were frozen at –80°C. Each
568 time point was performed in triplicated. The cells were freeze-thawed for three cycles,
569 harvested, and centrifuged at 900 × *g* for 5 min at 4°C. The supernatant was recovered and
570 activated with 80 µg/ml of trypsin for 30 min at 37°C. Two-fold serial dilutions were
571 prepared and used to determine the viral titers described previously (59, 81).

572 **Fluorescence labeling of purified rRV**. 100 µl of purified and trypsin activated biotinylated
573 rRV/VP4-BAP was incubated with 1 µl of streptavidin-Alexa Fluor 555 (2mg/ml)
574 (ThermoFisher Scientific) for 1 h at room temperature. The tube was snaped every 20 min.
575 Unbound streptavidin and streptavidin conjugated to rRV/VP4-BAP were separated by
576 loading the 50 µl reaction mixture on top of 100 µl of a 20% sucrose-PBS cushion followed
577 by centrifugation for 40 min at 20 psi in an air-driven ultracentrifuge (Airfuge, Beckman
578 Coulter). Pellet was resuspended in 20 µl TBS (25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 5 mM
579 KCl, 1 mM $MgCl_2$, 0.7 mM $CaCl_2$, 0.7 mM Na_2HPO_4, 5.5 mM dextrose).

580 **Immunofluorescence.** For virus internalization experiments, 1 µl of rRV particles
581 conjugated to StAV-Alexa555 diluted in 50 µl of DMEM was adsorbed over MA104 cells for

582 15 min and kept on a metal tray cooled to -20°C. Cells were then transferred to 37°C and
583 fixed at the indicated time-points with ice-cold methanol for 3 min on dry ice.
584 For later times post-infection, the virus was adsorbed for 1 h at 4˚C in a reduced
585 volume. Then, cells were transferred to 37˚C and treated at the indicated time points with
586 100 µM biotin in serum-free DMEM. Cells were fixed when indicated in 2%
587 paraformaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature or
588 in ice cold-methanol for 3 min at -20°C.
589 VLS experiments were performed as described by Buttafuoco *et al*. (90). In
590 experiments using the inhibitors, the drug was added at 1 hpi and maintained until 6 hpi.
591 The concentrations used 0.5 µM jasplakinolide (61, 70), 10 µM cytochalasin B (92) and 5 µM
592 blebbistatin (63) were described elsewhere.
593 All immunofluorescence assays were processed as described by Buttafuoco *et al*. (90).
594 Images were acquired using a confocal laser scanning microscope (CLSM) (DM550Q; Leica).
595 Data were analyzed with the Leica Application Suite (Mannheim, Germany) and Image J
596 (93).
597 **LLPS assay.** MA/NSP2-mCherry cells were seeded at a density of 1.2 x 10^4 cells per well 8-
598 wells Lab-Tek® Chamber Slide™ (Nunc, Inc. Cat #177402). For RV infection, the virus was
599 adsorbed at MOI of 25 VFU/cell diluted in 30 µl of serum-free DMEM, incubated at 4˚C for 1
600 h in an orbital shaker, and then volume filled to 100µl with serum-free DMEM followed by
601 incubation at 37˚C. At 5 hpi, the medium was replaced by medium containing 3.5% 1,6-
602 hexanediol in 2% FCS-DMEM and cells were incubated for 6 min at 37˚C. Then the drug was
603 washed out by removing the medium, washing the cells three times with PBS, adding fresh
604 2% FCS-DMEM, and incubating at 37˚C. At the designated time post-recovery, cells were
605 fixed with 2% PFA for 10 min at room temperature. Finally, nuclei were stained by

606 incubating cells with 1 µg/ml of DAPI (4',6-diamidino-2-phenylindole) in PBS for 15 min at
607 room temperature. Samples were mounted in ProLong™ Gold antifade mountant
608 (ThermoFisher Scientific), and images were acquired using a fluorescence microscope
609 (DMI6000B, Leica). Data were analyzed using ImageJ software (version 2.1.0/1.53;
610 https://imagej.net/Fiji).

611 **Quantification of viroplasms.** The number of viroplasms was acquired and analyzed as
612 previously described (25, 61, 92). Data analysis was performed using Microsoft® Excel for
613 Mac version 16.58. Statistical analysis, unpaired parametric Welch's t-test comparison post-
614 test, and plots were performed using Prism 9 for macOS version 9.3.1 (GraphPad Software,
615 LLC).

616 **Rotavirus electropherotype**. Rotavirus genome extraction and visualization were
617 performed as previously described (94). Briefly, MA104 cells at a density of 3 x 10^5 cells per
618 well in a 6-well multiwell plate were infected with a virus at MOI of 10 VFU/cell and
619 incubated in 1 ml serum free DMEM until complete cytopathic effect was reached. The cells
620 and supernatant were harvested, followed by three cycles of liquid nitrogen freeze and 37°C
621 water bath. Then, the samples were mixed vigorously at a ratio of 1:1 with saturated phenol
622 solution pH 4.3 (Merck) and centrifuged for 15 min at 13'000 rpm. The aqueous phase was
623 recovered, and the previous step was repeated. Then, the RNA in the recovered aqueous
624 phase was precipitated by mixing with 0.1 vol 3M Na Acetate at pH 5.2 and 2 vol of 100%
625 ethanol. Samples were incubated for 30 min at -80°C and then centrifuged at 13'000 rpm
626 for 30 min and 4°C. The pellet was resuspended in 15 µl distilled water and mixed with 10 µl
627 Gel Loading dye 6X (New England BioLabs). The samples were migrated in an 8.5% SDS-
628 polyacrylamide gel at 180 Volts for 120 min, followed by staining with GelRed® Acid gel
629 Stain (Biotium) for 30 min. Images were acquired at Odyssey® FC (LI-COR Biosciences).

630 **Transmission electron microscopy.** MA104 cells were seeded at 1×10^5 cells in a 2-cm^2 well
631 onto sapphire discs and infected with either rRV/wt or rRV/VP_4-BAP at an MOI of 50
632 VFU/ml. At 6 and 12 hpi, the sapphire discs were collected, fixed with 2.5% glutaraldehyde
633 in 100 mM Na/K-phosphate buffer, pH 7.4, for 1 h at 4°C and then kept in 100 mM Na/K-
634 phosphate buffer overnight at 4°C. Afterward, samples were postfixed in 1% osmium
635 tetroxide in 100 mM Na/K-phosphate buffer for 1 h at 4°C, dehydrated in a graded ethanol
636 series starting at 70%, followed by two changes in acetone, and embedded in Epon.
637 Ultrathin sections (60 to 80 nm) were cut and stained with uranyl acetate and lead citrate.

638 For staining biotinylated TLPs with streptavidin-gold, purified particles were
639 dialyzed overnight at 4˚C in TNC buffer (10 mM Tris-HCl, pH 7.5, 140 mM NaCl, 10mM
640 $CaCl_2$). The TLPs were adsorbed for 10 min on carbon-coated Parlodion films mounted on
641 300-mesh copper grids (EMS). Samples were washed once with water, fixed in 2.5%
642 glutaraldehyde in 100 mM Na/K-phosphate buffer, pH 7.0, for 10 min at room temperature,
643 and washed twice with PBS before incubation for 2 h at room temperature with 10 µl
644 streptavidin conjugated to 10 nm colloidal gold (Sigma-Aldrich, Inc). Before use, the
645 streptavidin-gold conjugate was treated as described previously to separate unconjugated
646 streptavidin from streptavidin-conjugated to colloidal gold (95). The viral particles were
647 washed three times with water and stained with 2% phosphotungstate, pH 7.0, for 1 min at
648 room temperature. Samples were analyzed in a transmission electron microscope (CM12;
649 Philips, Eindhoven, The Netherlands) equipped with coupled device (CCD) cameras
650 (Ultrascan 1000 and Orius SC1000A; Gatan, Pleasanton, CA, USA) at an acceleration
651 voltage of 100 kV.

652 For calculation of the diameter of virus particles by negative staining, the area of each
653 virus particle was calculated using Imaris software (version 2.1.0/1.53c; Creative Commons

654 license) and then converted to the diameter as follows: $d = 2 \times \sqrt[2]{A/\pi}$, where A is the area
655 and d is the diameter of the particle, respectively.

656 **siRNA reverse transfection.** For silencing gs4 of SA11 strain, the following siRNA pool:
657 siVP4-25 (5'-uugcucacgaauucuuauatt-3'), siVP4-931 (5'-gaaguuaccgcacauacuatt-3') and
658 siVP4-1534 (5'-auugcaaugucgcaguuaatt-3') was designed and synthesized by Microsynth
659 AG (Switzerland). siRNA-A (sc-37007, Santa Cruz Biotechnology) was used as negative
660 siRNA control. siRNA reverse transfection was performed by mixing 1.2 µl siRNA 5 µM with
661 1 µl lipofectamine RNAiMAX transfection reagent (Invitrogen, ThermoFisher Scientific) to a
662 final volume of 100 µl with Opti-MEM® (Gibco, ThermoFisher Scientific) in a well of 24-well
663 plate and incubated for 20 min at room temperature. To reach a 10 nM siRNA final
664 concentration, 2 x 10^4 cells diluted in 500 µl DMEM supplemented with 10% FCS were
665 added on top. Samples were incubated for 60 h prior to analysis. Thus, cells were infected
666 with simian RV strain SA11 at MOI 12 VFU/cell as described previously (25, 90, 91).

667 **FRAP.** 1.2 x 10^4 MA/NSP2 cells per well were seeded in µ-Slide 18-well glass-bottom plates
668 (Ibidi). Cells were RV-infected at MOI of 15 VFU/cell and kept in DMEM-SF. At 4.5 hpi, the
669 cells were counterstained with Hoechst 33342 diluted in FluoroBRITE DMEM (Gibco,
670 Cat.No. A18967-01) at a concentration of 1 µg/ml, incubated for 30 min at 37°C, and
671 subjected to FRAP analysis. FRAP experiments were performed with an SP8 Falcon
672 confocal laser scanning microscope (CLSM) from Leica equipped with a 63x objective (NA
673 1.4) using the FRAP function of the LasX software (Leica) as follows: a circular area of 2 µm
674 in diameter, encompassing an entire viroplasm, was bleached with the 405 nm and 481 nm
675 lasers simultaneously, each at 100% laser power, for 20 iterations. The fluorescent recovery
676 was monitored by taking fluorescence images of the mCherry channel every 2 seconds for

677 140 seconds. For each FRAP acquisition, a circular area of 2 µm, encompassing an entire
678 unbleached viroplasm in the same cell, was used as the fluorescent control, and a squared
679 area (5 µm x 5 µm) outside of a cell was chosen as the background. The entire FRAP dataset
680 was analyzed with MatLab (MATLAB R2020b, Mathworks) using the FRAP-tool source
681 code from easyFRAP (Cell Cycle Lab, Medical School, University of Patras). Fully
682 normalized data were used to generate FRAP diagrams and calculate the recovery half-
683 times (T-half) and mobile fractions from independent measurements. Representative
684 images were taken and processed for each FRAP experiment using the Imaris software v9.5
685 (Bitplane, Oxford Instruments). Fluorescent intensities of FRAP movies were normalized
686 using a customized Fiji pipeline (93).

687 **Quantitative RT-PCR**. MA104 cells at a density of 5 x 10^5 cells per well in 6 well multiwell
688 plates were RV-infected at an MOI of 18 VFU/cell. The virus was adsorbed for 1 h at 4°C. At
689 4 hpi, RNA was extracted using a quick RNA miniprepPlus kit (Zymo Research) according to
690 the manufacturer's instructions. cDNA synthesis was prepared with 1 µg of RNA using an
691 AMV reverse transcription system (Promega) and random primers according to the
692 manufacturer's instructions. Then 2 µl of 1:10 diluted cDNA was mixed with 0.25 µl forward
693 primer (10 pmol/µl), 0.25 µl reverse primer (10 pmol/µl) (**Supplementary Table 2**), 10 µl
694 SYBR™ Green PCR master mix (Applied biosystems), and 7.5 µl nuclease-free water
695 followed by incubation at QuantStudio3 (Applied Biosystems, ThermoFisher Scientific)
696 using standard amplification protocol with an annealing temperature of 60 °C. The relative
697 expression of genes was calculated with the formula $2^{-\Delta Ct}$, where ΔCt = Ct target gene- Ct
698 endogenous control gene. HPRT-1, SDHA and GAPDH were used as an endogenous control
699 housekeeping gene. Data were analyzed using Microsoft Excel for MAC (version 16.58).

Statistical analysis and plots were done using Prism9 for macOS (version 9.3.1) (GraphPad Software, LLC).

Purification and transfection of DLPs. RV amount sufficient to infect 1 x 10^6 cells at MOI of 15 VFU/cell was diluted up to 110 µl with TBS. Then EDTA pH 8.0 was added to a final concentration of 10 mM. The samples were incubated for 1 h at 37°C with gentle mixing and centrifuged at 3000 rpm for 2 min. The supernatant was recovered, loaded on top of a 100 µl cushion composed of 20% sucrose in PBS, and ultracentrifuged at 20 psi for 60 min on an air-driven ultracentrifuge (Airfuge, Beckman Coulter). For quality control, DLPs were monitored by negative staining electron microscopy. The DLPs were normalized using Pierce™ Coomassie protein assay kit (ThermoFisher Scientific) to determine the amount of total protein followed by an indirect ELISA to normalize to rotavirus protein using as primary antibody a guinea pig anti-rotavirus, which detected mainly VP6. Thus, DLPs were transfected by diluting them in 12.5 µl Opti-MEM and mixed with 0.75 µl Lipofectamine 2000 (Invitrogen) in 12.5 µl Opti-MEM. The mixture was incubated for 20 min at room temperature and added onto 1 x 10^4 MA104 cells per well in a 96-well black wall tissue culture plate (Greiner). At 6 hpi, cells were fixed with paraformaldehyde and prepared for immunofluorescence as described above. Images were acquired using a CLSM and then processed with Image J2 version 2.3.0/1.53f.

In-cell western assay. RV-infected MA104 cells (1 x 10^4) were seeded in a 96-well black tissue culture plate (Greiner). At indicated times post-infection, cells were fixed with 2% paraformaldehyde in PBS for 10 min at room temperature, followed by permeabilization with 0.1% triton X-100-PBS for 10 min at room temperature. Cells were blocked with 2% BSA in PBS for 1 h at room temperature and then incubated with primary antibodies diluted

723 in blocking buffer for 1 h at room temperature in the shaker. The cells were incubated with
724 the corresponding secondary antibody conjugated to IRDye® 800 CW (LI-COR). The cell
725 signal was normalized using CellTag™ 700 stain (LI-COR) diluted at 1:800. The cells were
726 washed three times with 0.01% Tween 20 in PBS between incubations. Samples were
727 acquired using an Odyssey CLx (LI-COR) followed by data analysis and normalization in
728 Microsoft® Excel for Mac version 16.58. Statistical analysis, unpaired Student's t-test, and
729 plots were performed using Prism 9 for macOS version 9.3.1 (GraphPad Software, LLC).

730 **Virus attachment assay.** Rotavirus binding was determined by a nonradioactive binding
731 assay as described previously in detail by Zarate *et al.* (56). The plates were coated with
732 goat anti-rotavirus (diluted 1:5000) to capture the virus. In addition, guinea pig anti-
733 rotavirus (diluted 1:5000) or streptavidin-HRP (diluted 1:500, Sigma) were used to detect
734 the virus. The reaction was developed using 100 µl of Pierce TMB substrate kit
735 (ThermoFisher) and stopped with 100 µl 1M H_2SO_4. Samples were recorded at an
736 absorbance of 450 nm using an Infinite M Plex (Tecan) plate reader. Data analysis was
737 performed using Microsoft® Excel for Mac version 16.58. Statistical analysis, semilog line
738 nonlinear regression, and plots were performed using Prism 9 for macOS version 9.3.1
739 (GraphPad Software, LLC).
740 **Linear peptide synthesis**. The linear peptide was synthesized using an automated peptide
741 synthesis system developed in our laboratory. In here, 200 mg of resin was swelled in 6 ml
742 100% dimethylformamide (DMF) for 5 min at 60 ºC with nitrogen bubbling using a Teflon
743 tube of 1/8-inch inner diameter. Importantly, the bubbling with nitrogen is kept during the
744 entire synthesis process. The Fmoc-protecting groups of the resin were removed by
745 washing twice with 6 ml solution of 20 % piperidine in DMF. For the first deprotection, the

746 sample was incubated for 1 min at 60 °C followed by aspiration. The second deprotection
747 was obtained by incubating for 4 min at 60 °C followed by four washes with 7 ml DMF. The
748 coupling of amino acids to unprotected resin or to the subsequent unprotected amino acids
749 in the elongated peptide coupled to the resin was performed by adding twice 7.5 ml
750 mixture of respective amino acid (5 eq/amine – 3 ml of 200mM), OxymaPure (7.5 eq./amine
751 – 2ml of) and DIC (10 eq./amine – 2.5ml of) dissolved in DMF. The amino acids were coupled
752 for two times 8 min at 60 °C with two times with 7 ml DMF wash after the first coupling and
753 three times with 7 ml DMF wash after the second coupling. After each coupling, the newly
754 bound amino acid was deprotected as mentioned above. The coupling reaction time was
755 specifically extended to 10 min for Asp, Glu and Arg and temperature was lowered to 50 °C
756 for Asp and Glu. The final deprotection was performed in the same manner as explained
757 above. The newly synthesized peptide was washed twice with methanol and dried under
758 vacuum. The peptide cleavage was carried out using TFA/TIS/H2O (94:5:1 v/v/v) for 4 h in 10
759 ml plastic syringe mounted on a rotter. Then, the peptide was collected in 50 ml tubes,
760 initially with gravity flow and subsequently, by inserting the plunger into the syringe. After
761 filtration, the peptide was precipitated with 50 ml ice cold tert-butylmethylether (TBME),
762 centrifuged at 4400 rpm for 15 min, and washed twice with TBME. For purification of the
763 crude peptide, it was dissolved in 10 ml of a solution containing100% mQ-H_2O, 0.1% TFA,
764 subjected to preparative RP-HPLC and obtained as TFA salt after lyophilisation. MS spectra
765 were provided by Mass Spectrometry of the Department of Chemistry and Biochemistry at
766 the University of Bern.
767
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Figure Legends

Figure 1. Generation of VP4-BAP tagged recombinant rotavirus (a) Schematic representation of BAP tag inserted into the lectin domain loops of the VP8* subunit of VP4 from RV simian strain SA11 (GenBank: X14204.1). The lysine (K, red) indicates the biotinylation site of BirA ligase. Four different VP4 proteins tagged with BAP (VP4-BAP) were built between amino acid regions T96-R101 (blue), E109-S114 (orange), N132-Q137 (pink), and K145-G150 (green). VP4 trimer ribbon structure is presented for the visualization of VP8* (yellow) and VP5CT (body and stalk, red) fragments. An inset in VP8* indicates the different positions in the hydrophobic loops of VP8* where the BAP tags were inserted and colored in blue, orange, pink, and green. **(b)** Immunoblot retardation assay of cell lysates transiently expressing wtVP4 and VP4-BAP, tagged at blue, orange, pink, and green positions, respectively. Untreated (-) and streptavidin-treated (+) samples are indicated. The membrane was incubated with anti-VP4 to detect unbound VP4-BAP and VP4-BAP bound to streptavidin (VP4-BAP•StAv). α-tubulin was used as a loading control**. (c)** dsRNA electropherotype of the genome segments of rRV/wt and rRV/VP4-BAP. The red arrow points to gs4-BAP. **(d)** Sequence chromatogram of gs4 of rRV/VP4-BAP. The sequence indicates the position of the linkers and the BAP tag in between the VP8*. **(e)** Immunoblotting of uninfected (lanes 1 and 4) or infected cell lysates in MA-cytBirA cells (left panel) or MA104 cells (right panel) infected with either rRV/wt (lanes 2 and 5) or rRV/VP4-BAP (lanes 3 and 6) [MOI, 25 VFU/cell]. Biotinylated proteins were detected with StAv-HRP. α-tubulin was used as a loading control. The red arrow and red star indicate biotinylated VP4-BAP and host undetermined biotinylated protein, respectively. **(f)** Immunoblot retardation assay of MA-cytBirA cell lysates infected with rRV/wt or rRV/VP4-BAP untreated or treated with StAv. The membrane was incubated with anti-VP4 and anti-

VP6 for detection of the virus. GAPDH was used as a loading control. The percentage of biotinylated VP_4 and VP_4-BAP was normalized to VP6 expression. **(g)** Visualization at a high resolution of purified virions isolated of rRV/VP_4-BAP infected MA-cytBirA cells untreated (-biotin, upper panel) or treated (+biotin, lower panel) with 100µM biotin. After purification, the virions were labeled with streptavidin conjugated to colloidal gold (12 nm), followed by negative staining electron microscopy (right panel). The scale bar is 100 nm. **(h)** Detection of purified unbiotinylated (grey bars) and biotinylated (open bars) rRV/VP_4-BAP particles. The particles were coated in an ELISA plate followed by binding to streptavidin-HRP or guinea pig anti-rotavirus followed by anti-guinea pig conjugated to HRP. The median from three independent experiments is shown (ordinary one-way ANOVA, (****) p-value<0.0001). **(i)** Scatter dot plot comparing the diameter of purified particles from unbiotinylated (-biotin, filled green dots) or biotinylated (+biotin, open green dots) RV/VP_4-BAP and rRV/wt (filled grey dots). The median value is indicated, n>40 particles, ordinary one-way ANOVA, (*) p-value<0.05 and (**) p-value<0.01.

Figure 2. RV protein localization upon rRV/VP_4-BAP infection. (a) rRV/wt (top panel) and rRV/VP_4-BAP (bottom panel) infected MA-cytBirA cells were untreated (-biotin) or treated with biotin (+biotin). Cells were fixed with paraformaldehyde at 6 hpi and stained for the detection of viroplasms (anti-NSP_5, green) and biotinylated proteins (streptavidin-Alexa 555, red). Nuclei were stained with DAPI (blue). **(b)** Immunostaining images of rRV/VP_4-BAP infected MA-cytBirA cells in the presence of biotin. At 6 hpi, paraformaldehyde-fixed cells were stained for the detection of VP_4-BAP (StAv, red) in viroplasms (anti-NSP_5, green) (upper row) or mature RV particles (anti-VP7 clone 159, green) (bottom row). **(c)** Immunofluorescence images of cells infected with rRV/wt (upper row) or rRV/VP_4-BAP (lower row) comparing the localization of VP_4 and VP_4-BAP (anti-VP_4, red), respectively.

The viroplasms were detected with anti-NSP_5 (green). Nuclei were stained with DAPI. In all the figures, the dashed white boxes correspond to the image insets of the right columns. The yellow arrows point to the VP_4-BAP or VP_4 signal. The scale bar is 10 μm.

Figure 3. Replication fitness of rRV/VP_4-BAP is delayed. (a) Virus replication fitness curve from 0 to 48 hpi of rRV/wt and rRV/VP_4-BAP. The curve represents the mean of three independent experiments. Welch's t-test, (*), $p<0.05$; (***), $p<0.001$ and ns, not significant. Quantification of cells with viroplasms of MA104 cells infected rRV/wt and rRV/VP_4-BAP at **(b)** same MOI (25 VFU/cell) or **(c)** the same numbers of virus particles.

Figure 4. Virus attachment and internalization of RV/wt and rRV/VP_4-BAP are comparable. (a) Virus attachment to MA104 cell surface with different amounts of purified rRV/wt (open grey dots) and rRV/VP_4-BAP (full green dots). Samples were detected using a guinea pig anti-RV. The result corresponds to the mean of nine normalized independent experiments. Data analysis corresponds to nonlinear regression, $p=0.2987$, $n=9$. Virus attachment assay to MA104 cell surface with different amounts of unbiotinylated (-biotin, full green dots) and biotinylated (+biotin, green open dots) purified rRV/VP_4-BAP detected with **(b)** guinea pig anti-RV followed by secondary antibody conjugated to HRP. Data analysis corresponds to nonlinear regression, $p=0.6151$, $n=6$. **(c)** Same as above, but samples were detected with streptavidin-HRP. Data analysis corresponds to nonlinear regression, $p<0.0001$, $n=6$. **(d)** Internalization of purified virions into MA104 cells at 0 min (upper panel) and 2 min (lower panel). Purified virions of rRV/wt and biotinylated rRV/VP_4-BAP were previously labeled with StAv-Alexa 555 (red). At the indicated time post-infection, cells were fixed and immunostained for VP7 trimers (mAb anti-VP7 clone 159, pink) and MTs (anti- α-tubulin, green). Nuclei were stained with DAPI (blue). White open boxes indicate the magnified images at the right. Arrows point to virus particle clumps

detected by anti-VP7. The scale bar is 20 µm. **(e-g)** Plots from quantitative RT-PCR comparing rotavirus transcripts of NSP5**,** VP6**,** and housekeeping gene (SDHA) in non-infected (NI)**,** rRV/wt- and rRV/VP4-BAP-infected cellular extracts at 4 hpi. The results correspond to the mean ± SEM of three independent experiments**,** ordinary one-way ANOVA**,** (**)**,** p<0.01**;** (***)**,** p<0.001**;** (****)**,** p<0.0001.

Figure 5. Viroplasm morphology and behavior for rRV/wt and rRV/VP4-BAP. (a) Representative high-resolution electron micrograph of viroplasms from rRV/wt (upper row) and rRV/VP4-BAP (lower row) infected MA104 cells at 6 hpi (left panel) and 12 hpi (right panel). Scale bars are 200nm and 500 nm**,** as indicated. **(b)** Fluorescence images of FRAP measurement of single viroplasm of cells infected with rRV/VP4-BAP (top) and rRV/wt (bottom) at pre-bleach**,** post-bleach**,** and recovery time conditions. Each inset indicates the bleached viroplasm of the images at the right. Nuclei were stained with Hoechst 33342. The scale bar is 10 µm. **(c)** FRAP recovery curve of NSP2-mCherry in single viroplasm from rRV/VP4-BAP (green) and rRV/wt (grey) infected MA/NSP2-mCherry cells at 5 hpi (n=27 and 25**,** respectively). Plots indicating recovery half-time **(d)** and diffusion **(e)** means of NSP2-mCherry in single viroplasms of rRV/VP4-BAP and rRV/wt after photobleaching.

Figure 6. Transfection of rRV/VP4-BAP DLPs is inefficient in forming viroplasms. (a) Size plot of TLPs and DLPs purified from rRV/wt and rRV/VP4-BAP. The median value is indicated**,** n>30 particles**,** ordinary one-way ANOVA**,** (*) p-value<0.05 and (****) p-value<0.0001. **(b)** In-cell western blot of MA104 cells transfected for 4 h with identical numbers of rRV/wt and rRV/VP4-BAP DLPs. RV infection was detected by staining with anti-NSP5 (IDye800**,** red). The loading control corresponds to cell tag 700 (green). The merge of the two channels is shown at the bottom. **(c)** Plot showing normalized amounts of NSP5 in rRV/wt and rRV/VP4-BAP-DLPs transfected MA104 cells. Each experiment was

done in triplicate. **(d)** Micrograph of rRV/wt and rRV/VP4-BAP DLPs transfected MA104 cells immunostained to detect viroplasms with anti-NSP5 (green). Nuclei were stained with DAPI (blue). Open dashed white box shows enlarged viroplasm inset at the right. The scale bar is 10 µm. **(e)** Quantification of the cells showing viroplasms upon transfection with DLPs of rRV/wt and rRV/VP4-BAP. The data correspond to the mean ± SD, Welch's t-test, (****), p>0.0001.

Figure 7. VP4 has a role in viroplasm formation. (a) Schematic representation for the characterization of LLPS condensates in viroplasms of RV-infected cells. At 5 hpi, RV-infected MA/NSP2-mCherry cells were treated with 1,6-HD for 6 min. The drug was washed out, and samples were fixed and imaged for viroplasm quantification at 0-, 2-, 15- and 30-min post-recovery. **(b)** Representative images of MA/NSP2-mCherry cells infected at 5 hpi with rRV/wt (upper row) or rRV/VP4-BAP (lower row) and treated with 3.5% of 1,6-HD. Cells were washed and monitored for viroplasm formation at 0-, 2-, 15- and 30-min post-recovery. White arrows point to cells with recovered viroplasms. The scale bar is 10 µm. Plots indicating the ratio of cells with viroplasms (**c**) and viroplasm counts per cell (**d**) normalized at initial conditions (5 hpi). **(e)** Immunoblot of cellular extracts prepared at 6 hpi from non-infected or SA11-infected MA104 or MA/NSP2-mCherry cells silenced with siVP4 or control siRNA (scr). The membrane was stained with anti-VP4, anti-NSP5, and anti-GAPDH (loading control). **(f)** Representative images of SA11-infected MA/NSP2-mCherry cells knocked down with scr (upper row) or siVP4 (lower row) and treated for 6 min with 3.5% of 1,6-HD. Cells were washed and monitored for viroplasm formation at 0-, 2-, 15- and 30-min post-recovery. White arrows point to cells showing recovered viroplasms. The scale bar is 10 µm. Plot indicating the ratio of cells with viroplasms (**g**) and viroplasm counts per cell (**h**) normalized to initial conditions after recovery from 1,6-HD treatment of SA11-

1159 infected MA104 cells silenced with siVP4 or scr The data represent the mean ± SEM
1160 Student's t-test (*), p<0.05; and (***), p<0.001.

1161 **Figure 8. VP4-BAP does not bind to the actin cytoskeleton. (a)** Immunostaining of
1162 MA104 cells showing NSP5 and NSP2 VLSs co-expressed with GFP (top panel), VP4-GFP
1163 (middle panel), and VP4-BAP-GFP (bottom panel). Cells were fixed with paraformaldehyde
1164 and immunostained to detect VLSs (anti-NSP5, red). Nuclei were stained with DAPI (blue).
1165 The scale bar is 10 µm. **(b)** Plot indicating the number of VLSs per cell when co-expressed
1166 with GFP, VP4-GFP, and VP4-BAP-GFP.The data represent the mean ± SEM, one-way
1167 ANOVA, (*), p<0.05 and (**), p<0.001. **(c)** Immunostaining of MA104 cells expressing VP4-
1168 GFP and VP4-BAP-GFP after treatment for 1 h before fixation without (-NOC) or with
1169 (+NOC) 10 µM nocodazole. Cells were fixed with methanol at 24 hpt and immunostained
1170 for GFP detection (anti-GFP, green) and actin cytoskeleton (anti-actin, red). Nuclei were
1171 stained with DAPI (blue). The scale bar is 10 µm. **(d)** Plot for the quantification of VP4-GFP
1172 and VP4-BAP-GFP filament lengths associated with actin filaments in cells untreated or
1173 treated with nocodazole. The data represent the mean± SD, Welch's t-test, (****),
1174 p<0.0001.

1175 **Figure 9. rRV/VP4-BAP impaired association with the actin cytoskeleton. (a)**
1176 Immunostaining of non-infected and SA11-infected MA104 cells. At 6 hpi, cells were fixed
1177 with methanol and immunostained to detect viroplasms (anti-NSP5, green) and actin
1178 cytoskeleton (anti-actin, cyan). Nuclei were stained with DAPI (blue). The scale bar is 20
1179 µm. Immunostaining of non-infected and rRV/wt or rRV/VP4-BAP-infected MA104 cells. At
1180 6 hpi, cells were fixed with methanol and immunostained for detection of **(b)** VP4 (anti-
1181 VP4, green) and actin cytoskeleton (anti-actin, cyan) and **(c)** VP4 (anti-VP5 clone 4G2, cyan)
1182 and myosin motor (anti-myosin IIa, green). Nuclei were stained with DAPI (blue). The scale

bar is 20 µm. Open yellow and red arrowheads point to stress fibers in the actin cytoskeleton and VP4 fibers, respectively. **(d)** Immunofluorescence of rRV/wt and rRV/VP4-BAP-infected MA104 cells treated at 1 hpi with either 0.5 µM jasplakinolide (jasp; middle panel) or 10 µM cytochalasin B (cyt B; lower panel). At 6 hpi, cells were fixed and immunostained to detect viroplasms (anti-NSP5, green). Nuclei were stained with DAPI (blue). The dashed open box corresponds to the inset of the enlarged image at the right. The scale bar is 10 µm. Plot for the quantification of **(e)** the ratio of cells showing viroplasms and **(f)** the number of viroplasms per cell. **(g)** Immunofluorescence of rRV/wt and rRV/VP4-BAP- infected MA104 cells untreated or treated at 1 hpi with 5 µM blebbistatin (BLB). At 6 hpi, cells were fixed and immunostained to detect viroplasms (anti-NSP5, green). Nuclei were stained with DAPI (blue). The dashed open box is an inset of the enlarged image at the right. The scale bar is 10 µm. Plot for the quantification of **(h)** the ratio of cells showing viroplasms and **(i)** the number of viroplasms per cell.

Figure 10. A peptide mimicking loop K145-G150 rescues the rRV/VP4-BAP phenotype. **(a)** Immunofluorescence images at 17 hpt of BHK-T_7 cells expressing VP4-GFP or VP4-BAP-GFP, untreated or treated with 25 µM SPL added immediately after transfection. Before methanol fixation, cells were treated for 1 h with 10 µM nocodazole. The cells were immunostained for GFP (anti-GFP, red), and nuclei were stained with DAPI (blue). Scale bar is 10 µm. **(b)** Plot quantifying the length of VP4 filaments untreated or treated with SPL as described in **(a)**. Welch ANOVA test, (**), $p< 0.01$ and (****), $p<0.0001$. **(c)** Representative immunofluorescence microphotograph of viroplasms of rRV/wt and rRV/VP4-BAP -infected cells untreated (-SPL) or treated (+SPL) at +1 and +3 hpi with 25 µM of SPL, as indicated. Viroplasms were immunostained at 6 hpi with anti-NSP5 (red), and nuclei were stained with DAPI (blue). The dashed white boxes correspond to the enlarged picture in the right

columns. Scale bar is 10 µm. Quantification plots for the ratio of cells with viroplasms **(d)** and the numbers **(e)** and size **(f)** of viroplasms per cell of MA104 cells infected with the indicated virus and untreated (UT) or treated with 25 µM SPL at +1 and +3 hpi. The data represent the mean ± SEM using Welch's ANOVA test, (***), $p<0.001$ and (****), $p<0.0001$. **(g)** Plot determining the virus progeny of rRV/wt and rRV/BAP infected cells untreated or treated at 1 hpi with 25 µM SPL. The data represent the mean +SEM of three independent experiments. Welch ANOVA test, (*) $p<0.05$.