

1 Identification of Rab18 as an Essential Host Factor for BKPyV Infection Using a Whole Genome RNA
2 Interference Screen

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25 **Abstract**

26

27 BK polyomavirus (BKPyV) is a human pathogen first isolated in 1971. BKPyV infection is ubiquitous in the
28 human population, with over 80% of adults worldwide being seropositive for BKPyV. BKPyV infection is
29 usually asymptomatic; however, BKPyV reactivation in immunosuppressed transplant patients causes
30 two diseases, polyomavirus-associated nephropathy and hemorrhagic cystitis. To establish a successful
31 infection in its host cells, BKPyV must travel in retrograde transport vesicles to reach the nuclei. To make
32 this happen, BKPyV requires the cooperation of host cell proteins. To further identify host factors
33 associated with BKPyV entry and intracellular trafficking, we performed a whole-genome siRNA screen
34 on BKPyV infection of primary human renal proximal tubular epithelial cells. The results revealed the
35 importance of the Ras-related protein Rab18 and syntaxin 18 for BKPyV infection. Our subsequent
36 experiments implicated additional factors that interact with this pathway, and suggest a more detailed
37 model of the intracellular trafficking process, indicating that BKPyV reaches the ER lumen through a
38 retrograde transport pathway between the late endosome and the ER.

39

40 **Keywords**

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42 siRNA screen, Rab18, syntaxin 18, NRZ complex, BKPyV

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46 **Introduction**

47 BK polyomavirus (BKPyV) is a small icosahedral DNA virus measuring approximately 45 nm in diameter
48 and was first isolated in 1971 (1). Subsequent serology surveys have revealed that up to 80% of the
49 world's population has been infected with BKPyV (2), and most of the initial infections occur in early
50 childhood (3). After initial exposure, BKPyV establishes an asymptomatic infection in the urinary tract
51 with periodic shedding into the urine (4). BKPyV reactivation when the immune system is compromised
52 in transplant patients, however, causes two diseases: polyomavirus-associated nephropathy (PVAN) and
53 hemorrhagic cystitis (HC) (5). PVAN is one of the leading causes of graft failure after kidney
54 transplantation (6). Also, BKPyV can be detected in the urine of 90% of allogeneic hematopoietic cell
55 transplant patients who suffer from HC, according to a recent study (7). Even though BKPyV was initially
56 isolated more than 45 years ago, the choices for clinical management of BKPyV reactivation are limited.
57 The first-line treatment for PVAN is lowering the dosage of immunosuppressants, which inevitably
58 increases the risk of acute rejection and graft failure (6). Other options for treating BKPyV reactivation
59 are treatment with cidofovir, leflunomide, and fluoroquinolones; however, the benefit of using these
60 drugs in addition to reducing immunosuppressants has been questioned and has not been investigated
61 carefully in randomized studies (6, 8). Due to the lack of specific antiviral medicines, management of
62 BKPyV reactivation remains a challenge.

63 BKPyV does not encode any polymerases. As a result, BKPyV exclusively relies on the host DNA
64 replication machinery for its genome replication. To establish a successful infection, BKPyV must deliver
65 its DNA genome into the nucleus to access the host DNA replication machinery, which means crossing
66 the plasma membrane, the ER membrane, and the nuclear envelope (9). Also, BKPyV needs to navigate
67 a crowded cytoplasm to reach the ER. Without any cooperation from host factors, this process would
68 seem difficult if not impossible.

69 Few host factors have previously been identified that participate in viral entry and intracellular
70 trafficking. To initiate infection, BKPyV binds to gangliosides GD1b or GT1b on the host cell
71 membrane(10). After binding to the cell membrane, BKPyV had been thought to enter host cells via a
72 caveolin-mediated pathway (11), however, subsequent experiments showed that caveolin was
73 dispensable for infecting human renal proximal tubule cells (12). After endocytosis, BKPyV enters the
74 endosome in the same way as other polyomaviruses (13-16). The acidification and maturation of the
75 endosome are essential for BKPyV infection (17), and activates a sorting machinery that involves Rab5,
76 Rab7, Rab9, and Rab11 proteins (14, 15, 18). After sorting through the late endosome, vesicles that

77 contain BKPyV traffic along microtubules and reach the ER at 8-12 hours post infection (17, 19). ER
78 lumen proteins are essential for polyomavirus disassembly and egress from the ER (9, 17, 20-22). Briefly,
79 protein disulfide isomerases induce capsid conformational changes and minor capsid protein exposure
80 (17, 23-28). These exposed minor proteins insert into the ER membrane (29, 30), and partially
81 disassembled polyomaviruses penetrate the ER membrane and enter the cytosol through the
82 endoplasmic reticulum-associated degradation pathway (9, 20, 21, 31, 32). In the cytosol, the nuclear
83 localization signal of the minor capsid proteins guides polyomaviruses into the nucleus via the importin
84 α/β pathway (33-35). In addition to this productive model of entry, a significant portion of internalized
85 polyomavirus enters a non-productive pathway (36). Distinguishing the critical events that BKPyV takes
86 to establish successful infection from other pathways that BKPyV takes remains a challenge.

87 Numerous assays have been developed for the purpose of identifying host factors associated with viral
88 infections, including genome-wide siRNA screening. By silencing every single human gene with an siRNA
89 pool, and then assessing the effects of the knockdown on infection, it is feasible to dissect the functions
90 of individual host proteins during the viral life cycle. siRNA screening has been extensively applied to
91 research on viruses, such as HIV (37), West Nile Virus (38), HPV (39), VSV (40), and another
92 polyomavirus, SV40 (41). However, no genome-wide siRNA screen has been reported for BKPyV.

93 Using a whole genome siRNA screen, we have identified a series of potential host factors that are
94 involved in BKPyV infection. DNAJ B14, which has previously been implicated in BKPyV entry, is our top
95 hit, and DNAJ B12 is also among our top 100 hits (21, 41). Most of the other hits we have identified have
96 not been previously reported, however, and many of them are involved in vesicular transport. In this
97 report, we present data showing that two of our primary hits, Rab18 and syntaxin 18, as well as two
98 members of the NRZ complex (RAD50 interactor 1 and ZW10 kinetochore protein), which have
99 previously been shown to interact with Rab18 and syntaxin 18, are essential host factors for BKPyV
100 infection.

101 **Results**

102 A high-throughput siRNA screen on BKPyV based on cell cycle analysis

103 To identify host factors involved in viral entry and intracellular trafficking, we developed and
104 implemented a high-throughput whole-genome siRNA screen for BKPyV infection in its natural host cell,
105 primary human renal proximal tubule epithelial (RPTE) cells. In this screen, BKPyV infection was
106 challenged with the Dharmacon siGENOME Smartpool siRNA library, which includes more than 18,000

107 human siRNA pools. Each pool contains four unique siRNAs that target the same host gene. The screen
108 was performed in triplicate. Every screen plate included the siGENOME non-targeting control (NTC) as a
109 negative control and a synthetic siRNA which corresponds to the natural BKPyV 5p miRNA targeting the
110 large tumor antigen (TA_g) mRNA (siTA_g) (42), as a positive control.

111 Our lab previously showed that BKPyV induces G2/M arrest to take full advantage of the host DNA
112 replication machinery (43); this provided us with a cost-efficient approach to evaluate viral infection in
113 siRNA-transfected cells. By applying a Hoechst DNA stain, cells in G2/M phase are distinguishable from
114 the rest of the cells based on the difference in DNA content in each nucleus. The percentage of G2/M
115 arrested cells (G2%) dramatically increases after BKPyV infection, and we adopted it as the readout for
116 the screening assay.

117 For the primary screen, RPTe cells were transfected with the siRNA library and cultured for 48 hours to
118 allow depletion of the targeted proteins. Next, cells were infected with BKPyV and cultured for an
119 additional two days. The cells were fixed and stained with Hoechst, and the numbers of nuclei in the
120 different stages of the cell cycle were quantified, recorded, and uploaded to the MScreen web tool
121 developed by the Center for Chemical Genomics (University of Michigan) (44). G2% values were
122 calculated using the MScreen web tool. To proceed with the data analysis, we scaled data from 0 to 100,
123 with 100 representing the effect of our positive control, and 0 representing the effect of negative
124 control. The effect of each siRNA pool was then calculated using this index scale. A larger index value
125 represents a stronger inhibitory effect on the BKPyV infection when an siRNA pool is introduced into
126 RPTe cells, which suggests that the targeted host factor is more likely to be required for BKPyV infection.

127 Z-factor is a statistical parameter widely used for screen assay evaluation. A minimal Z-factor of 0.5 is
128 required for a reliable siRNA screen (45). The overall Z-factor achieved in our whole primary screen was
129 0.6, and the R-squared values were approximately 0.9 between replicates. We compared the data from
130 the three replicates in a pairwise manner, as illustrated in the scatter plots in Figure 1. 70% of the
131 siRNA pools in the siGENOME siRNA library inhibited BKPyV infection to some degree when compared to
132 the non-targeting siRNA control. A similar distribution of inhibitory effects was also observed in an siRNA
133 screen using the siGENOME library to assess cellular genes involved in HPV infection (39). The results of
134 the primary screen are in Supplemental Table 1, Sheet1.

135 We parsed the candidate genes into functional categories and pathways using the Database for
136 Annotation, Visualization, and Integrated Discovery (DAVID) enrichment analysis tool (46, 47). The

137 DAVID analysis showed that translation-related genes were the most enriched group of genes that is
138 required for viral infection among the candidate genes (Supplemental Table 1, Sheet2). Considering that
139 BKPvV exclusively relies on the host protein synthesis machinery, this result is consistent with our
140 expectation. Other than translation-related genes, several clusters of genes that are required for viral
141 infection were also significantly enriched, especially budding and coat proteins associated with vesicular
142 transport. Based on the enrichment analysis results, we manually selected 147 siRNA pools for
143 secondary validation.

144 G2% is an indirect readout for BKPvV infection. Knocking down cell cycle regulatory proteins may also
145 affect G2/M arrest independent of viral infection; thus false positive or false negative candidates could
146 be introduced into the results during the primary screen. To eliminate these false candidates, we next
147 applied a direct readout, immunofluorescent staining for the early viral protein TAg, to validate these
148 147 genes. RPTE cells were transfected and infected in 96 well plates with a subset of the siGENOME
149 library that contains the selected 147 siRNA pools. Infected cells were fixed at 48 hours post infection
150 and probed for TAg with primary antibody and FITC-labeled secondary antibody. After acquiring images,
151 the integrated TAg fluorescent intensity per nucleus was recorded, and the inhibition index was
152 calculated as in the primary screen (Supplemental Table 2).

153 After the secondary screen, DNAJ B14, which has previously been implicated in BKPvV entry, was our
154 top hit, and DNAJ B12 and DNAJC3, which are also involved in polyomavirus trafficking, were also among
155 our top ten hits (21, 41), which indicates the overall robustness of our screen. Rab18 was one of our top
156 validated hits other than these DNAJ proteins. Moreover, syntaxin 18, a protein that has previously been
157 demonstrated to interact with Rab18, also passed our validation.

158

159 The Rab18/NRZ/syntaxin 18 complex is required for BKPvV infection

160 The Rab protein family is a group of small GTPases that regulate membrane trafficking, and that have
161 been implicated in polyomavirus intracellular trafficking (14, 18, 48). Rab18, however, had not
162 previously been associated with polyomavirus infection. The Rab18 interaction network has been
163 thoroughly investigated (49), and more than 40 proteins have been identified as interacting with Rab18.
164 Among these proteins, syntaxin 18 was also one of our top hits in the screen.

165 To determine whether Rab18 and syntaxin 18 are required during BKPvV infection, RPTE cells in 12 well
166 plates were transfected with pooled or individual siRNAs targeting these proteins. Cells transfected with

167 the NTC siRNA pool and siTag served as negative and positive controls, respectively. After transfection,
168 cells were cultured for two days to allow for depletion of the targeted proteins, and then infected with
169 BKPvV at a multiplicity of infection (MOI) of 1 infectious unit (IU)/cell. Cells were lysed at 48 hours post
170 infection, and expression of the targeted proteins, TAg, and β -actin or glyceraldehyde 3-phosphate
171 dehydrogenase (GAPDH) were assessed by Western blotting (Figure 2A). The choice of β -actin or GAPDH
172 was based on avoiding co-migration of the loading control with the protein of interest. Knockdown of
173 Rab18 and syntaxin 18 reduced TAg expression, indicating that both proteins are required for efficient
174 infection. Knockdown of Rab18 entirely blocked BKPvV infection. While there was only partial
175 knockdown of syntaxin 18 with both the pooled or individual siRNAs, TAg expression decreased
176 correspondingly. Because syntaxin 18 is a SNARE protein (50), this suggests that BKPvV enters the ER
177 lumen via a vesicle fusion step.

178 In addition to syntaxin 18, Rab18 interacts with ZW10 kinetochore protein (ZW10) and RAD50 Interactor
179 1 (RINT1), which together with neuroblastoma amplified gene (NAG) are members of the NRZ complex
180 (51). Upon activation by GTP, Rab18 interacts with the ZW10 kinetochore protein from the NRZ
181 complex, thereby forming a Rab18/NRZ/syntaxin 18 complex at the ER (49). In this complex, syntaxin 18
182 functions as a t-SNARE on the ER membrane where the NRZ components work as a tether to assist with
183 retrograde vesicle docking (51, 52). NRZ/syntaxin 18 cooperate in capturing Rab18-labeled vesicles and
184 initiating the fusion process on the ER membrane. Although none of the NRZ complex proteins were
185 identified in our primary screen, we hypothesized that they are required for BKPvV infection due to their
186 ability to interact with both Rab18 and syntaxin 18. To test whether NRZ components are involved in
187 BKPvV infection, we knocked down RINT1 and ZW10, followed by infection. We could not test NAG due
188 to lack of a useful antibody to measure its expression. The Western blot results indicate that disrupting
189 the NRZ complex by knocking down these two components interferes with infection (Figure 2B).

190

191 Rab18 colocalizes with the viral capsid during BKPvV intracellular trafficking

192 We next asked whether Rab18 is associated with BKPvV-containing vesicles, since Rab18 targets vesicles
193 to the ER (53, 54). To test this, we fixed RPTE cells at 6 and 8 hours post-infection and stained for the
194 BKPvV major capsid protein, VP1, and Rab18. Images of the stained RPTE cells were acquired with a
195 confocal microscope. The confocal images show that Rab18 and VP1 are partially colocalized at 6 and 8
196 hours post-infection (Figure 3). This is consistent with the asynchronous trafficking of BKPvV from

197 endosomes after entry (17). These results support our hypothesis that BKPv travels in Rab18-positive
198 vesicles after being sorted through endosomes.

199

200 BKPv traffics differently after Rab18, syntaxin 18, or NRZ knockdown

201 To establish a successful infection, BKPv must reach the ER lumen where disassembly occurs (17). After
202 internalization into host cells, conformational changes of the capsid are neither required nor observed
203 before polyomaviruses enter the ER lumen (27, 28). When polyomavirus particles reach the ER lumen,
204 luminal enzymes disrupt the disulfide bonds between VP1 monomers, thereby initiating disassembly of
205 the viral capsid (26). This process in the ER can be visualized by assaying for VP1 monomer, dimer, and
206 oligomer bands after separation of proteins under non-reducing conditions by SDS-PAGE (17, 28). To
207 confirm that BKPv requires Rab18 to reach the ER lumen and initiate disassembly, we examined BKPv
208 disassembly after Rab18 knockdown. RPTe cells were transfected with NTC or Rab18 siRNA for 48 hours
209 and infected with BKPv at MOI 5. Protein samples were processed and assayed at 24 hours post
210 infection using Western blotting of reducing and non-reducing gels (17, 28). At 0 hours post infection,
211 most of the BKPv particles were intact and too large to enter the non-reducing SDS-PAGE gel, and no
212 VP1 monomers (42 kDa) or dimers were visible. At 24 hours post infection in cells in which Rab18 was
213 present, BKPv began to disassemble, and VP1 monomers, dimers, and oligomers could be detected.
214 However, the non-reducing gel suggests that the BKPv particle could not disassemble efficiently
215 without Rab18 (Figure 4A, top panel). The reducing gel shows that equal amounts of VP1 are detectable
216 in the presence or absence of Rab18, indicating that knocking down Rab18 did not prevent BKPv from
217 attaching to or entering RPTe cells. We also assessed the effect of knocking down RINT1, ZW10, and
218 syntaxin 18 on BKPv disassembly (Figure 4A, bottom panel). In each case, lower amounts of VP1
219 monomers and dimers were detected in the non-reducing gel, suggesting that BKPv cannot
220 disassemble efficiently without the help of the NRZ complex.

221 Previous studies showed that mouse polyomavirus (MPyV) could enter cells that do not produce proper
222 ganglioside receptors; afterward, these MPyV particles are trapped in a dead-end pathway and cannot
223 establish a successful infection. This indicates that polyomaviruses enter both productive and non-
224 productive pathways inside host cells. These non-productive pathways result in a diffuse distribution of
225 viral particles as detected by immunofluorescent staining (36). In the presence of proper ganglioside
226 receptors, polyomaviruses leave the cell periphery, move along microtubules, and reach the perinuclear

227 area (17, 19, 55, 56). Ultimately, the productive pathway leads to the ER (17, 28, 57). Since we saw that
228 BKPyV could not reach the ER lumen without Rab18 while the entry of BKPyV was not affected (Figure
229 4A), we wished to address if BKPyV traffics in a different pattern under Rab18 knockdown. To test this,
230 Rab18 was depleted as previously described, and RPTE cells were infected with BKPyV at MOI of 5. Cells
231 were fixed and stained for VP1 at 24 hours post infection (Figure 4B). In the presence of Rab18, BKPyV
232 forms bright foci in the perinuclear area as previously observed (9). However, Rab18 knockdown caused
233 dispersion of the bright perinuclear VP1 foci, and the staining of VP1 appeared to be diffuse and spread
234 throughout the cytoplasm (Figure 4B). Thus, without Rab18 BKPyV traffics in a pattern that is suggestive
235 of a non-productive pathway (36).

236

237 BKPyV is enriched in the late endosome without Rab18.

238 Our previous study demonstrated that acidification of the endosome is essential for BKPyV infection
239 (17), and MPyV colocalizes with the late endosome marker Rab7 after entry (18). Furthermore, BKPyV
240 cannot traffic efficiently to the ER and initiate capsid disassembly without abundant Rab18 and instead
241 appears to be trapped in an unknown compartment(s) diffusely located in the cytoplasm (Figure 4). To
242 identify this compartment, we knocked down Rab18 in RPTE cells, fixed cells at 24 hours post infection,
243 and probed for various organelle markers (Figure 5). We found that the diffuse viral particles do not
244 colocalize with the early endosome marker EEA1, cis-Golgi marker GM130 (58), or trans-Golgi marker
245 Golgin 97 (59). On the other hand, we found that the VP1-rich area overlaps with Rab7. To further
246 confirm this colocalization, images of VP1 and Rab7 were taken using confocal microscopy (Figure 6).
247 We found that the dispersed VP1 partially colocalizes with Rab7 when Rab18 is knocked down,
248 suggesting some viral particles are trapped in the late endosome in the absence of Rab18. There is also
249 some VP1 that does not colocalize with Rab7. This may be a result of asynchronous trafficking of BKPyV,
250 and additional intermediate and dead-end compartments could also exist during the retrograde
251 trafficking step between the endosome and the ER. Our results suggest that BKPyV reaches ER lumen
252 through a Rab18-mediated retrograde transport pathway between the late endosome and the ER.

253

254 **Discussion**

255 BK polyomavirus (BKPyV) was initially isolated more than 45 years ago (1), however, our understanding
256 of the early events in the BKPyV life cycle contains gaps. Polyomaviruses appear to take advantage of

257 multiple endocytic pathways to enter the host cell. However, some of the pathways that BKPyV uses are
258 non-productive (36), which suggests that identifying host factors solely by morphological observations,
259 especially protein co-localization evidence, may be deceiving. SV40, BKPyV, MPyV, and cholera toxin
260 have been observed to enter the cell via a caveolin-mediated pathway (11, 60-64). However, subsequent
261 experiments have demonstrated that caveolin is dispensable (12, 14, 65-68). Because of the limitations
262 of the available biological tools, research of the earliest stages of the polyomavirus life cycle has
263 progressed relatively slowly.

264 Whole genome siRNA screening provides an unbiased tool to investigate some of the remaining
265 questions. However, one of the biggest challenges of developing an siRNA screen assay is finding a
266 readout that is sensitive, cost efficient, and suitable for high throughput automation. One of the most
267 common readouts for a viral siRNA screen is cell viability. However, BKPyV, along with many viruses,
268 does not lyse host cells within a reasonable period compared to the duration of the RNAi effect in cells.
269 This behavior of BKPyV makes cell viability measurements impractical. Another common strategy to
270 evaluate viral infection is by incorporating reporter genes into the viral genome. Because BKPyV is highly
271 sensitive to genomic modification, several approaches attempting to integrate reporter genes into the
272 BKPyV genome have failed (data not shown). Therefore, constructing modified viral particles containing
273 a reporter gene was not practical for us. Finally, immunofluorescent staining for viral protein expression
274 is neither cost- nor time-efficient for processing hundreds of plates. For all these reasons, we needed a
275 different readout for evaluating viral infection.

276 The percentage of cells in G2/M phase provided us a cost-efficient assay for the primary screen. Our
277 primary screen results indicated that knocking down 70% of the human genes in RPTE cells inhibited
278 BKPyV infection to some degree when compared to the non-targeting control. A similar data distribution
279 was also observed in an siRNA screen for HPV infection using the same siRNA library (39). We found that
280 the NTC siRNA reproducibly slightly increased BKPyV infection compared to the no siRNA control. This
281 could be because of interference of exogenous siRNA with the host intrinsic miRNA processing and
282 silencing mechanism. BKPyV encodes a miRNA that downregulates TAg mRNA levels (42, 69). If the
283 introduction of exogenous siRNA affects the miRNA processing and silencing mechanism, TAg expression
284 would increase due to lower levels of the mature BKPyV miRNA.

285 The increased BKPyV infection induced by the non-targeting siRNA control could also be an off-target
286 effect of RNAi. While validating our primary screening results, we found many of our primary hits were
287 not reproducible when tested with individual siRNAs instead of siRNA pools. It appears that off-target

288 effects of RNAi are inevitable for whole genome siRNA screens, and an ‘ideal non-targeting’ siRNA pool
289 does not exist (70). The most likely cause of the off-target effect is the passenger strand of the siRNA.
290 Each siRNA carries a complimentary strand (passenger strand) to enhance the stability of the siRNA
291 molecules. This passenger strand can also be integrated into the RNA-induced silencing complex (RISC),
292 which possibly increases off-target effects. Moreover, even if only the guide strand enters the RISC
293 complex, RISC prefers the 2nd to the 8th nucleotides of that strand, called the seed region, to recognize
294 targeted mRNAs (70-74). Considering this characteristic of RNAi, each seven-nucleotide seed region can
295 target more than one gene. Off-target effects can therefore increase the expense of validation and
296 lower the efficiency of screening.

297 After validating some of the top hits as well as other genes that are known to be involved in the same
298 vesicular trafficking pathway, Rab18, syntaxin 18, ZW10, and RINT1 were confirmed as essential host
299 factors for BKPyV infection. Rab18 has been associated with lipid droplet homeostasis and vesicular
300 transport between the Golgi apparatus and the ER (53, 54). The majority of Rab18 usually localizes to
301 the membrane of the ER and the cis-Golgi apparatus (53). Also, Rab18 is recruited to the surface of lipid
302 droplets, the endosome, and the lysosome (75, 76). Rab18 has been shown to interact with syntaxin 18,
303 ZW10, and RINT1 by affinity chromatography followed by mass spectrometry (49). Based on previous
304 studies on yeast proteins, ZW10 and RINT1 interact with each other via their N-termini and interact with
305 the NAG protein to form the NRZ complex. The t-SNARE protein syntaxin 18 on the ER membrane
306 indirectly attaches to the NRZ complex (51, 52, 77). The overall structure of the NRZ complex is a
307 protrusion about 20 nm in length from the surface of the ER membrane (78). GTP-activated Rab18 on
308 the surface of vesicles interacts with ZW10, thereby tethering vesicles to the ER membrane. Syntaxin 18
309 is a SNARE protein and mediates vesicle fusion to the ER (50). After the NRZ complex captures vesicles,
310 syntaxin 18 mediates vesicle fusion with the ER membrane.

311 We propose a model of BKPyV entry and intracellular trafficking to the ER based on previous findings
312 and our current results (Figure 7). To initiate infection, BKPyV first binds to its ganglioside receptors
313 GT1b and GD1b (10). After initial attachment, BKPyV is believed to form deep invaginations of the host
314 plasma membrane in the same manner as SV40 (79). The length of ceramide tails of the ganglioside
315 plays a critical role in formation of the invagination (79). Next, BKPyV enters host cells via a ganglioside-
316 dependent, caveolin- and clathrin-independent pathway (12, 80). Meanwhile, glycoproteins on the
317 plasma membrane appear to serve as traps for polyomavirus (81): viral particles that bind to
318 glycoproteins will eventually enter non-productive pathways. Only those particles that bind to the

319 correct ganglioside receptor can enter the productive infection pathway (36). Furthermore, binding to
320 the gangliosides is sufficient to drive transport of artificial particles to the ER (18). This indicates that
321 binding to the proper ganglioside is both necessary and sufficient for BKPvV to traffic in retrograde
322 transport vesicles to the ER. In addition, conformational changes of the capsid are neither required nor
323 observed before polyomaviruses enter the ER lumen (27, 28). These findings reveal that BKPvV may
324 remain bound to the membrane throughout the intracellular trafficking until reaching the ER lumen.
325 BKPvV enters the endosome in the same manner as other polyomaviruses (13-15). Acidification of the
326 endosome activates a ganglioside-sorting machinery that sorts BKPvV into secondary vesicles along with
327 gangliosides (17, 18, 82). Without Rab18, we found that BKPvV accumulates in the late endosome
328 (marked by Rab7). This suggests that Rab18 mediates late endosome to ER trafficking of BKPvV and that
329 it buds and traffics together with virus-containing vesicles along microtubules (17, 19, 55, 83). After the
330 NRZ complex on the ER membrane captures and tethers vesicles to the ER surface, syntaxin 18 further
331 interacts with a yet to be identified v-SNARE on the vesicles, and the syntaxin 18/v-SNARE complex
332 mediates vesicle fusion to the ER membrane. This allows successful entry of BKPvV into the ER lumen.
333 Our finding supports the conclusion that late endosome to ER trafficking plays a critical role in BKPvV
334 infection.

335 It has been shown that fluorescently labeled gangliosides can spontaneously transport in a retrograde
336 direction to the ER (77). BKPvV and the other polyomaviruses are therefore like hitchhikers that take a
337 ride along this ganglioside-mediated retrograde pathway to the ER. Whether additional interactions
338 between the viral capsid and host proteins play a role in BKPvV trafficking to the ER is still unclear.
339 Gangliosides play important roles in both endocytosis and retrograde trafficking. Polyomaviruses and
340 several toxins are proposed to take advantage of lipid-mediated endocytosis and retrograde trafficking
341 pathways (reviewed by Ewers and Helenius (80)). Beside polyomaviruses, some other non-enveloped
342 viruses infect cells in a similar manner; therefore, they may also utilize part of this lipid-mediated
343 retrograde trafficking pathway to establish an infection (84): The retrograde paths of both HPV and
344 BKPvV can be blocked with retrograde traffic inhibitors retro-1, retro-2, and BFA (17, 39, 85, 86).
345 Norovirus also binds to gangliosides (87, 88), and forms deep invaginations on model membranes (89).
346 Gangliosides have been demonstrated to be important for rotavirus infection (90), and rotavirus also
347 traffics to and is sorted through the endosome (91). However, details of the Rab18-mediated pathway
348 are largely unknown, and further efforts will be needed to fully reveal the details of this retrograde
349 trafficking pathway.

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359

360 **Materials and Methods**

361 Cell culture. Primary renal proximal tubule epithelial (RPTE) cells purchased from Lonza were maintained
362 in the recommended medium, REGM BulletKit (REGM/REBM, Lonza, CC-3190), at 37°C with 5% CO₂ in a
363 humidified incubator. Cells recovered from a single frozen vial from Lonza (Passage 2) were cultured for
364 three passages. Afterward, cells at passage 5 were passaged one more time and plated for the
365 screening, or aliquoted and frozen in liquid nitrogen for later experiments. For all experiments other
366 than the primary screen, frozen aliquots (Passage 5) were recovered about one week before each
367 experiment, and cells were then plated for experiments.

368 Infection. BKPyV (Dunlop) was cultured, purified on a cesium chloride linear gradient, and titered as
369 described previously (17, 92). RPTE cells were infected as follows at two days post siRNA transfection.
370 Cells were pre-chilled for 15 min at 4°C. Purified viruses were diluted to 175,000 IU/ml (MOI 1) or
371 875,000 IU/ml (MOI 5) in REBM/REGM. 400 µl of the diluted virus were added to the wells of a 12 well
372 plate and incubated at 4°C for 1 hour with shaking every 15 minutes to distribute the inoculum over the
373 entire well. The plate was transferred to 37°C after the 1-hour incubation.

374 siRNA and siRNA library. The whole-genome human siGENOME smart pool siRNA library from
375 Dharmacon was acquired and prepared by the Center for Chemical Genomics (University of Michigan).
376 All other siRNAs were also purchased from Dharmacon: Non-targeting siRNA control (D-001206-14);
377 Rab18 siRNA (LQ-010824-00); STX18 siRNA (LQ-020624-01); ZW10 siRNA (LQ-003948-00); RINT1 siRNA
378 (LQ-004976-01); siRNA targeting large T antigen (custom synthesized with the sequence 5'
379 AUCUGAGACUUGGGAAGAGCAU 3'), which corresponds to the natural BKPyV 5p miRNA (42).

380 Primary siRNA screening in 384 well plates. The siRNA library was rehydrated at 500 nM in siRNA buffer
381 (Dharmacon, B-002000-UB-100) according to the Basic siRNA Resuspension protocol from Dharmacon. 1
382 μ l 500nM siRNA suspension was spotted into each well of 384-well PE Viewplates on a Biomek
383 laboratory automation workstation. RPTE cells were transfected according to the Lipofectamine
384 RNAiMAX (Thermo Fisher Scientific) manual. Briefly, transfection complexes were prepared by adding 9
385 μ l of diluted transfection reagent (0.78% RNAiMAX reagent v/v in REBM/REGM without antibiotics) to
386 each well of the 384-well plates onto which the siRNAs had been spotted. The transfection complexes
387 were incubated at room temperature for 20 min before adding 1,800 cells suspended in 10 μ l
388 REBM/REGM without antibiotics to each well. Transfected cells were cultured at 37°C for 48 h, after
389 which cells were infected by the following procedure: incubate plates at 4°C for 15 min; dilute purified
390 BKPyV Dunlop in cold REGM/REBM; dispense 5 μ l 1,800,000 IU/ml virus to each well with a Multidrop
391 Combi reagent dispenser (Thermo Fisher Scientific); incubate plates at 4°C for 1 hour; transfer plates to
392 37°C for additional 48 hours. Next, cells were fixed with 4% paraformaldehyde (Electron Microscopy
393 Sciences) at room temperature for 20 min, permeabilized with 0.1% Triton x-100 (MilliporeSigma) in PBS
394 for 5 min at room temperature, and stained with 2 μ g/ml Hoechst 33342 (Thermo Fisher Scientific,
395 H3570) in PBS for 15 min at room temperature. Cells were washed three times with PBS after each
396 staining step. Images of the wells were taken with an ImageXpress Micro XLS high-throughput
397 microscope and analyzed with MetaXpress High-Content Image Acquisition and Analysis software. The
398 quantified data generated from MetaXpress were uploaded and further analyzed with MScreen, a high-
399 throughput analysis system developed by the Center for Chemical Genomics (University of Michigan)
400 (44). Low-quality data points generated from wells with contamination or significant cell death were
401 discarded.

402 Secondary siRNA screening in 96 well plate. For siRNA transfection in 96 well plates, the primary
403 screening protocol was scaled up by applying 3 times the volume used in 384 wells plate. In addition to
404 the Hoechst stain, cells were also incubated for 1 hour each with 5% goat serum, anti-TAg monoclonal
405 antibody (pAb416) at 1:200 dilution in 5% goat serum (93), and goat anti-mouse IgG-FITC
406 (MilliporeSigma, F2012) at 1:200 dilution in 5% goat serum. Cells were rinsed with 1x PBS 3 times
407 between each step. Images of the wells were taken with an ImageXpress Micro XLS high-throughput
408 microscope and analyzed with MetaXpress High-Content Image Acquisition and Analysis software for
409 TAg intensity.

410 Tertiary validation in 12 well plates. siRNAs were rehydrated at 1 μ M in siRNA buffer (Dharmacon, B-
411 002000-UB-100). Transfection complexes were prepared by mixing 20 μ l of 1 μ M siRNA with 380 μ l of
412 diluted transfection reagent (0.74% RNAiMAX reagent v/v in REBM/REGM without antibiotics) in each
413 well of a 12-well plate. The transfection complexes were incubated at room temperature for 20 min
414 before adding 70,000 cells suspended in 400 μ l REBM/REGM without antibiotics to each well. RPTe cells
415 were infected at two days post transfection.

416 Preparation of protein lysates. Cells were lysed at 48 hours post infection with E1A buffer [50 mM HEPES
417 (pH 7), 250 mM NaCl, and 0.1% NP-40, with inhibitors: 5 μ g/ml PMSF, 5 μ g/ml aprotinin, 5 μ g/ml
418 leupeptin, 50 mM sodium fluoride and 0.2 mM sodium orthovanadate added right before use]
419 (MilliporeSigma). For non-reducing gels, cells were rinsed with 1x PBS with 10 mM N-Ethylmaleimide
420 (MilliporeSigma, E3876) and harvested by scraping. Harvested cells were further pelleted with a
421 centrifuge and lysed with a Triton lysis buffer [10 mM Tris (pH 7.6), 10 mM sodium phosphate, 130 mM
422 NaCl, 1% Triton X-100, 10 mM NEM, protease inhibitors (Roche, 11697498001)]. Insoluble cell debris
423 was removed by centrifuging lysed cells at 16,100 xg and discarding the pellet. Protein concentration
424 was quantified with the Bradford assay (Bio-Rad).

425 Western blotting. Protein samples were separated on 12% SDS-PAGE gels. After electrophoresis, the
426 proteins were transferred to a nitrocellulose membrane (MilliporeSigma, pore size 0.2 μ m) in Towbin
427 transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) at 60 V overnight. Membranes were
428 blocked with 2% nonfat milk in PBS-T buffer (144 mg/L KH_2PO_4 , 9 g/L NaCl, 795 mg/L Na_2HPO_4 , pH 7.4,
429 0.1% Tween 20) for 1 hour. Membranes were probed with primary and secondary antibodies diluted in
430 2% milk in PBS-T as follows: TAg (pAb416) at 1:5,000 dilution (93); syntaxin 18 (Abcam, ab156017)
431 diluted in 5% BSA at 1:1,000; Rab18 (MilliporeSigma, SAB4200173) at 1:10,000; VP1(pAb5G6) 1:1000;
432 GAPDH (Abcam, ab9484) at 1: 10,000; ZW10 kinetochore protein (Abcam, ab53676) at 1:300; RAD50
433 interactor 1 (MilliporeSigma, HPA019875) at 1:200; β -actin (Cell signaling, #4967) at 1:10,000;
434 horseradish peroxidase (HRP)-conjugated ECL sheep anti-mouse (GE healthcare, NA931V) at 1: 5,000;
435 and HRP-conjugated ECL donkey anti-rabbit antibody (GE Healthcare, NA934V) at 1: 5,000. Protein
436 bands were visualized with HRP substrate (Millipore, WBLUF0100) and exposure to X-ray film or the
437 Syngene PXi gel doc system.

438 Immunofluorescent staining. 18 mm circular coverslips (#1.5 thickness, Electron Microscopy Sciences,
439 72222) were coated with 0.1% poly-L-lysine in water (MilliporeSigma, P8920) in 12 well plates for 5 min
440 at room temperature. Coverslips were then rinsed with cell culture grade water and allowed to dry for

441 at least 2 hours. RPTE cells were then seeded onto the coverslips in the 12-well plate. For processing,
442 the cells were fixed with 4% PFA at room temperature for 20 min. Antigen was retrieved with antigen
443 retrieval buffer (100 mM Tris, 5% [w/v] urea, pH 9.5) at 95 °C for 10 min. Cell membranes were
444 permeabilized with 0.1% Triton X-100 in PBS at room temperature for 5 min. Coverslips were blocked
445 with 5% goat serum in PBS for 1 hour and probed with diluted primary and secondary antibody
446 successively, 1 hour each. Coverslips were washed with PBS for three times between each step. Lastly,
447 coverslips were mounted with Prolong Gold Reagent with DAPI (Thermo Fisher Scientific, P36931).
448 Primary or secondary antibodies were diluted in 5% goat serum (MilliporeSigma) as follows: VP1
449 (pAb5G6) at 1:200; Rab18 (MilliporeSigma, SAB4200173) at 1:1500; Rab7 (Cell Signaling, #9367) at
450 1:100; GM130 (Cell Signaling, #12480) at 1:200; EEA1 (Cell Signaling, #3288) at 1:200; Golgin-97 (Cell
451 Signaling, #13192) at 1:100; goat anti-mouse IgG–FITC (MilliporeSigma, F2012) at 1:200; goat anti-rabbit
452 IgG-DL594 (Thermo Fisher Scientific, 35561) at 1:200. Images were taken with Olympus BX41 or Leica
453 inverted SP5 confocal microscope system with a 100x objective. Confocal images were acquired and
454 processed with LAS AF, LAS X software from Leica.

455 **References**

- 456 1. Gardner SD, Field AM, Coleman DV, Hulme B. 1971. New human papovavirus (B.K.) isolated from
457 urine after renal transplantation. *The Lancet* 1:1253–1257.
- 458 2. Egli A, Infanti L, Dumoulin A, Buser A, Samaridis J, Stebler C, Gosert R, Hirsch HH. 2009.
459 Prevalence of Polyomavirus BK and JC Infection and Replication in 400 Healthy Blood Donors. *J*
460 *Infect Dis* 199:837–846.
- 461 3. Kean JM, Rao S, Wang M, Garcea RL. 2009. Seroepidemiology of human polyomaviruses. *PLoS*
462 *Pathog* 5:e1000363.
- 463 4. Ahsan N, Shah KV. 2006. Polyomaviruses and human diseases. *Adv Exp Med Biol* 577:1–18.
- 464 5. Jiang M, Abend JR, Johnson SF, Imperiale MJ. 2009. The role of polyomaviruses in human disease.
465 *Virology* 384:266–273.
- 466 6. Kuypers DRJ. 2012. Management of polyomavirus-associated nephropathy in renal transplant
467 recipients. *Nat Rev Nephrol* 8:390–402.
- 468 7. Lunde LE, Dasaraju S, Cao Q, Cohn CS, Reding M, Bejanyan N, Trottier B, Rogosheske J, Brunstein
469 C, Warlick E, Young JAH, Weisdorf DJ, Ustun C. 2015. Hemorrhagic cystitis after allogeneic
470 hematopoietic cell transplantation: risk factors, graft source and survival. *Bone Marrow*
471 *Transplant* 50:1432–1437.
- 472 8. Ramos E, Drachenberg CB, Wali R, Hirsch HH. 2009. The Decade of Polyomavirus BK-Associated
473 Nephropathy: State of Affairs. *Transplantation* 87:621–630.

- 474 9. Bennett SM, Jiang M, Imperiale MJ. 2013. Role of cell-type-specific endoplasmic reticulum-
475 associated degradation in polyomavirus trafficking. *J Virol* 87:8843–8852.
- 476 10. Low JA, Magnuson B, Tsai B, Imperiale MJ. 2006. Identification of gangliosides GD1b and GT1b as
477 receptors for BK virus. *J Virol* 80:1361–1366.
- 478 11. Moriyama T, Marquez JP, Wakatsuki T, Sorokin A. 2007. Caveolar endocytosis is critical for BK
479 virus infection of human renal proximal tubular epithelial cells. *J Virol* 81:8552–8562.
- 480 12. Zhao L, Marciano AT, Rivet CR, Imperiale MJ. 2016. Caveolin- and clathrin-independent entry of
481 BKPyV into primary human proximal tubule epithelial cells. *Virology* 492:66–72.
- 482 13. Querbes W, O'Hara BA, Williams G, Atwood WJ. 2006. Invasion of host cells by JC virus identifies
483 a novel role for caveolae in endosomal sorting of noncaveolar ligands. *J Virol* 80:9402–9413.
- 484 14. Liebl D, Difato F, Horníková L, Mannová P, Štokrová J, Forstová J. 2006. Mouse polyomavirus
485 enters early endosomes, requires their acidic pH for productive infection, and meets transferrin
486 cargo in Rab11-positive endosomes. *J Virol* 80:4610–4622.
- 487 15. Engel S, Heger T, Mancini R, Herzog F, Kartenbeck J, Hayer A, Helenius A. 2011. Role of
488 endosomes in simian virus 40 entry and infection. *J Virol* 85:4198–4211.
- 489 16. Ashok A, Atwood WJ. 2003. Contrasting roles of endosomal pH and the cytoskeleton in infection
490 of human glial cells by JC virus and simian virus 40. *J Virol* 77:1347–1356.
- 491 17. Jiang M, Abend JR, Tsai B, Imperiale MJ. 2009. Early events during BK virus entry and
492 disassembly. *J Virol* 83:1350–1358.
- 493 18. Qian M, Cai D, Verhey KJ, Tsai B. 2009. A lipid receptor sorts polyomavirus from the
494 endolysosome to the endoplasmic reticulum to cause infection. *PLoS Pathog* 5:e1000465.
- 495 19. Eash S, Atwood WJ. 2005. Involvement of cytoskeletal components in BK virus infectious entry. *J*
496 *Virol* 79:11734–11741.
- 497 20. Inoue T, Tsai B. 2015. A nucleotide exchange factor promotes endoplasmic reticulum-to-cytosol
498 membrane penetration of the nonenveloped virus simian virus 40. *J Virol* 89:4069–4079.
- 499 21. Bagchi P, Walczak CP, Tsai B. 2015. The Endoplasmic Reticulum Membrane J Protein C18
500 Executes a Distinct Role in Promoting Simian Virus 40 Membrane Penetration. *J Virol* 89:4058–
501 4068.
- 502 22. Chromy LR, Oltman A, Estes PA, Garcea RL. 2006. Chaperone-mediated in vitro disassembly of
503 polyoma- and papillomaviruses. *J Virol* 80:5086–5091.
- 504 23. Norkin LC, Anderson HA, Wolfrom SA, Oppenheim A. 2002. Caveolar Endocytosis of Simian Virus
505 40 Is Followed by Brefeldin A-Sensitive Transport to the Endoplasmic Reticulum, Where the Virus
506 Disassembles. *J Virol* 76:5156–5166.
- 507 24. Rainey-Barger EK, Mkrтчian S, Tsai B. 2007. Dimerization of ERp29, a PDI-like protein, is essential

- 508 for its diverse functions. *Mol Biol Cell* 18:1253–1260.
- 509 25. Schelhaas M, Malmström J, Pelkmans L, Haugstetter J, Ellgaard L, Grünewald K, Helenius A. 2007.
510 Simian Virus 40 depends on ER protein folding and quality control factors for entry into host cells.
511 *Cell* 131:516–529.
- 512 26. Walczak CP, Tsai B. 2011. A PDI family network acts distinctly and coordinately with ERp29 to
513 facilitate polyomavirus infection. *J Virol* 85:2386–2396.
- 514 27. Nelson CDS, Ströh LJ, Gee GV, O'Hara BA, Stehle T, Atwood WJ. 2015. Modulation of a pore in the
515 capsid of JC polyomavirus reduces infectivity and prevents exposure of the minor capsid proteins.
516 *J Virol* 89:3910–3921.
- 517 28. Inoue T, Dosey A, Herbstman JF, Ravindran MS, Skiniotis G, Tsai B. 2015. ERdj5 Reductase
518 Cooperates with Protein Disulfide Isomerase To Promote Simian Virus 40 Endoplasmic Reticulum
519 Membrane Translocation. *J Virol* 89:8897–8908.
- 520 29. Daniels R, Rusan NM, Wadsworth P, Hebert DN. 2006. SV40 VP2 and VP3 insertion into ER
521 membranes is controlled by the capsid protein VP1: implications for DNA translocation out of the
522 ER. *Molecular Cell* 24:955–966.
- 523 30. Rainey-Barger EK, Magnuson B, Tsai B. 2007. A chaperone-activated nonenveloped virus
524 perforates the physiologically relevant endoplasmic reticulum membrane. *J Virol* 81:12996–
525 13004.
- 526 31. Inoue T, Tsai B. 2011. A large and intact viral particle penetrates the endoplasmic reticulum
527 membrane to reach the cytosol. *PLoS Pathog* 7:e1002037.
- 528 32. Geiger R, Andrichke D, Friebe S, Herzog F, Luisoni S, Heger T, Helenius A. 2011. BAP31 and BiP
529 are essential for dislocation of SV40 from the endoplasmic reticulum to the cytosol. *Nature Cell
530 Biology* 13:1305–1314.
- 531 33. Nakanishi A, Shum D, Morioka H, Otsuka E, Kasamatsu H. 2002. Interaction of the Vp3 nuclear
532 localization signal with the importin $\alpha 2/\beta$ heterodimer directs nuclear entry of infecting simian
533 virus 40. *J Virol* 76:9368–9377.
- 534 34. Nakanishi A, Itoh N, Li PP, Handa H, Liddington RC, Kasamatsu H. 2007. Minor capsid proteins of
535 simian virus 40 are dispensable for nucleocapsid assembly and cell entry but are required for
536 nuclear entry of the viral genome. *J Virol* 81:3778–3785.
- 537 35. Bennett SM, Zhao L, Bosard C, Imperiale MJ. 2015. Role of a nuclear localization signal on the
538 minor capsid proteins VP2 and VP3 in BKPyV nuclear entry. *Virology* 474:110–116.
- 539 36. You J, O'Hara SD, Velupillai P, Castle S, Lavery S, Garcea RL, Benjamin T. 2015. Ganglioside and
540 Non-ganglioside Mediated Host Responses to the Mouse Polyomavirus. *PLoS Pathog*
541 11:e1005175.
- 542 37. Zhou H, Xu M, Huang Q, Gates AT, Zhang XD, Castle JC, Stec E, Ferrer M, Strulovici B, Hazuda DJ,
543 Espeseth AS. 2008. Genome-scale RNAi screen for host factors required for HIV replication. *Cell*

- 544 Host Microbe 4:495–504.
- 545 38. Krishnan MN, Ng A, Sukumaran B, Gilfoy FD, Uchil PD, Sultana H, Brass AL, Adametz R, Tsui M,
546 Qian F, Montgomery RR, Lev S, Mason PW, Koski RA, Elledge SJ, Xavier RJ, Agaisse H, Fikrig E.
547 2008. RNA interference screen for human genes associated with West Nile virus infection. *Nature*
548 455:242–245.
- 549 39. Lipovsky A, Popa A, Pimienta G, Wyler M, Bhan A, Kuruvilla L, Guie M-A, Poffenberger AC, Nelson
550 CDS, Atwood WJ, DiMaio D. 2013. Genome-wide siRNA screen identifies the retromer as a
551 cellular entry factor for human papillomavirus. *Proc Natl Acad Sci USA* 110:7452–7457.
- 552 40. Lee AS-Y, Burdeinick-Kerr R, Whelan SPJ. 2014. A genome-wide small interfering RNA screen
553 identifies host factors required for vesicular stomatitis virus infection. *J Virol* 88:8355–8360.
- 554 41. Goodwin EC, Lipovsky A, Inoue T, Magaldi TG, Edwards APB, Van Goor KEY, Paton AW, Paton JC,
555 Atwood WJ, Tsai B, DiMaio D. 2011. BiP and multiple DNAJ molecular chaperones in the
556 endoplasmic reticulum are required for efficient simian virus 40 infection. *MBio* 2:e00101–11.
- 557 42. Seo GJ, Fink LHL, O'Hara B, Atwood WJ, Sullivan CS. 2008. Evolutionarily conserved function of a
558 viral microRNA. *J Virol* 82:9823–9828.
- 559 43. Jiang M, Zhao L, Gamez M, Imperiale MJ. 2012. Roles of ATM and ATR-Mediated DNA Damage
560 Responses during Lytic BK Polyomavirus Infection. *PLoS Pathog* 8:e1002898.
- 561 44. Jacob RT, Larsen MJ, Larsen SD, Kirchoff PD, Sherman DH, Neubig RR. 2012. MScreen. *J Biomol*
562 *Screen* 17:1080–1087.
- 563 45. Zhang J, Chung T, Oldenburg K. 1999. A Simple Statistical Parameter for Use in Evaluation and
564 Validation of High Throughput Screening Assays. *J Biomol Screen* 4:67–73.
- 565 46. Da Wei Huang, Sherman BT, Lempicki RA. 2009. Systematic and integrative analysis of large gene
566 lists using DAVID bioinformatics resources. *Nat Protoc* 4:44–57.
- 567 47. Da Wei Huang, Sherman BT, Lempicki RA. 2008. Bioinformatics enrichment tools: paths toward
568 the comprehensive functional analysis of large gene lists. *Nucl Acids Res* 37:1–13.
- 569 48. Mannová P, Forstová J. 2003. Mouse polyomavirus utilizes recycling endosomes for a traffic
570 pathway independent of COPI vesicle transport. *J Virol* 77:1672–1681.
- 571 49. Gillingham AK, Sinka R, Torres IL, Lilley KS, Munro S. 2014. Toward a comprehensive map of the
572 effectors of rab GTPases. *Dev Cell* 31:358–373.
- 573 50. Inuma T, Aoki T, Arasaki K, Hirose H, Yamamoto A, Samata R, Hauri H-P, Arimitsu N, Tagaya M,
574 Tani K. 2009. Role of syntaxin 18 in the organization of endoplasmic reticulum subdomains. *J Cell*
575 *Sci* 122:1680–1690.
- 576 51. Tagaya M, Arasaki K, Inoue H, Kimura H. 2014. Moonlighting functions of the NRZ (mammalian
577 Dsl1) complex. *Front Cell Dev Biol* 2:25.

- 578 52. Hirose H, Arasaki K, Dohmae N, Takio K, Hatsuzawa K, Nagahama M, Tani K, Yamamoto A,
579 Tohyama M, Tagaya M. 2004. Implication of ZW10 in membrane trafficking between the
580 endoplasmic reticulum and Golgi. *EMBO J* 23:1267–1278.
- 581 53. Dejgaard SY, Murshid A, Erman A, Kizilay O, Verbich D, Lodge R, Dejgaard K, Ly-Hartig TBN,
582 Pepperkok R, Simpson JC, Presley JF. 2008. Rab18 and Rab43 have key roles in ER-Golgi
583 trafficking. *J Cell Sci* 121:2768–2781.
- 584 54. Liu S, Storrie B. 2012. Are Rab proteins the link between Golgi organization and membrane
585 trafficking? *Cell Mol Life Sci* 69:4093–4106.
- 586 55. Zila V, Difato F, Klimova L, Huerfano S, Forstová J. 2014. Involvement of microtubular network
587 and its motors in productive endocytic trafficking of mouse polyomavirus. *PLoS ONE* 9:e96922.
- 588 56. Sanjuan N, Porrás A, Otero J. 2003. Microtubule-dependent intracellular transport of murine
589 polyomavirus. *Virology* 313:105–116.
- 590 57. Magnuson B, Rainey EK, Benjamin T, Baryshev M, Mkrtchian S, Tsai B. 2005. ERp29 triggers a
591 conformational change in polyomavirus to stimulate membrane binding. *Molecular Cell* 20:289–
592 300.
- 593 58. Nakamura N. 1995. Characterization of a cis-Golgi matrix protein, GM130. *J Cell Biol* 131:1715–
594 1726.
- 595 59. Follit JA. 2006. The Intraflagellar Transport Protein IFT20 Is Associated with the Golgi Complex
596 and Is Required for Cilia Assembly. *Mol Biol Cell* 17:3781–3792.
- 597 60. Parton RG. 1994. Regulated internalization of caveolae. *J Cell Biol* 127:1199–1215.
- 598 61. Orlandi PA, Fishman PH. 1998. Filipin-dependent Inhibition of Cholera Toxin: Evidence for Toxin
599 Internalization and Activation through Caveolae-like Domains. *J Cell Biol* 141:905–915.
- 600 62. Richterová Z, Liebl D, Horák M, Palková Z, Stokrová J, Hozák P, Korb J, Forstová J. 2001. Caveolae
601 are involved in the trafficking of mouse polyomavirus virions and artificial VP1 pseudocapsids
602 toward cell nuclei. *J Virol* 75:10880–10891.
- 603 63. Pelkmans L, Kartenbeck J, Helenius A. 2001. Caveolar endocytosis of simian virus 40 reveals a
604 new two-step vesicular-transport pathway to the ER. *Nature Cell Biology* 3:473–483.
- 605 64. Eash S, Querbes W, Atwood WJ. 2004. Infection of vero cells by BK virus is dependent on
606 caveolae. *J Virol* 78:11583–11590.
- 607 65. Damm E-M, Pelkmans L, Kartenbeck J, Mezzacasa A, Kurzchalia T, Helenius A. 2005. Clathrin- and
608 caveolin-1-independent endocytosis: entry of simian virus 40 into cells devoid of caveolae. *J Cell*
609 *Biol* 168:477–488.
- 610 66. Gilbert JM, Goldberg IG, Benjamin TL. 2003. Cell Penetration and Trafficking of Polyomavirus. *J*
611 *Virol* 77:2615–2622.

- 612 67. Shogomori H, Futerman AH. 2001. Cholera toxin is found in detergent-insoluble rafts/domains at
613 the cell surface of hippocampal neurons but is internalized via a raft-independent mechanism. *J*
614 *Biol Chem* 276:9182–9188.
- 615 68. Torgersen ML, Skretting G, van Deurs B, Sandvig K. 2001. Internalization of cholera toxin by
616 different endocytic mechanisms. *J Cell Sci* 114:3737–3747.
- 617 69. Broekema NM, Imperiale MJ. 2013. miRNA regulation of BK polyomavirus replication during early
618 infection. *In*.
- 619 70. Tschuch C, Schulz A, Pscherer A, Werft W, Benner A, Hotz-Wagenblatt A, Barrionuevo LS, Lichter
620 P, Mertens D. 2008. Off-target effects of siRNA specific for GFP. *BMC Mol Biol* 9:60.
- 621 71. Jackson AL, Bartz SR, Schelter J, Kobayashi SV, Burchard J, Mao M, Li B, Cavet G, Linsley PS. 2003.
622 Expression profiling reveals off-target gene regulation by RNAi. *Nat Biotechnol* 21:635–637.
- 623 72. Jackson AL, Burchard J, Schelter J, Chau BN, Cleary M, Lim L, Linsley PS. 2006. Widespread siRNA
624 “off-target” transcript silencing mediated by seed region sequence complementarity. *RNA*
625 12:1179–1187.
- 626 73. Birmingham A, Anderson EM, Reynolds A, Ilesley-Tyree D, Leake D, Fedorov Y, Baskerville S,
627 Maksimova E, Robinson K, Karpilow J, Marshall WS, Khvorova A. 2006. 3' UTR seed matches, but
628 not overall identity, are associated with RNAi off-targets. *Nat Meth* 3:199–204.
- 629 74. Mohr S, Bakal C, Perrimon N. 2010. Genomic screening with RNAi: results and challenges. *Annual*
630 *review of biochemistry* 79:37–64.
- 631 75. Zhang J, Chang D, Yang Y, Zhang X, Tao W, Jiang L, Liang X, Tsai H, Huang L, Mei L. 2017.
632 Systematic investigation on the intracellular trafficking network of polymeric nanoparticles.
633 *Nanoscale* 9:3269–3282.
- 634 76. Zhang J, Zhang X, Liu G, Chang D, Liang X, Zhu X, Tao W, Mei L. 2016. Intracellular Trafficking
635 Network of Protein Nanocapsules: Endocytosis, Exocytosis and Autophagy. *Theranostics* 6:2099–
636 2113.
- 637 77. Civril F, Wehenkel A, Giorgi FM, Santaguida S, Di Fonzo A, Grigorean G, Ciccarelli FD, Musacchio
638 A. 2010. Structural analysis of the RZZ complex reveals common ancestry with multisubunit
639 vesicle tethering machinery. *Structure* 18:616–626.
- 640 78. Ren Y, Yip CK, Tripathi A, Huie D, Jeffrey PD, Walz T, Hughson FM. 2009. A structure-based
641 mechanism for vesicle capture by the multisubunit tethering complex Dsl1. *Cell* 139:1119–1129.
- 642 79. Ewers H, Römer W, Smith AE, Bacia K, Dmitrieff S, Chai W, Mancini R, Kartenbeck J, Chambon V,
643 Berland L, Oppenheim A, Schwarzmann G, Feizi T, Schwille P, Sens P, Helenius A, Johannes L.
644 2010. GM1 structure determines SV40-induced membrane invagination and infection. *Nature*
645 *Cell Biology* 12:11–8– sup pp 1–12.
- 646 80. Ewers H, Helenius A. 2011. Lipid-mediated endocytosis. *Cold Spring Harb Perspect Biol*
647 3:a004721–a004721.

- 648 81. Qian M, Tsai B. 2010. Lipids and proteins act in opposing manners to regulate polyomavirus
649 infection. *J Virol* 84:9840–9852.
- 650 82. Chinnapen DJ-F, Hsieh W-T, Welscher te YM, Saslowsky DE, Kaoutzani L, Brandsma E, D'Auria L,
651 Park H, Wagner JS, Drake KR, Kang M, Benjamin T, Ullman MD, Costello CE, Kenworthy AK,
652 Baumgart T, Massol RH, Lencer WI. 2012. Lipid sorting by ceramide structure from plasma
653 membrane to ER for the cholera toxin receptor ganglioside GM1. *Dev Cell* 23:573–586.
- 654 83. Moriyama T, Sorokin A. 2008. Intracellular trafficking pathway of BK Virus in human renal
655 proximal tubular epithelial cells. *Virology* 371:336–349.
- 656 84. Taube S, Jiang M, Wobus CE. 2010. Glycosphingolipids as receptors for non-enveloped viruses.
657 *Viruses* 2:1011–1049.
- 658 85. Nelson CDS, Carney DW, Derdowski A, Lipovsky A, Gee GV, O'Hara B, Williard P, DiMaio D, Sello
659 JK, Atwood WJ. 2013. A retrograde trafficking inhibitor of ricin and Shiga-like toxins inhibits
660 infection of cells by human and monkey polyomaviruses. *MBio* 4:e00729–13.
- 661 86. Carney DW, Nelson CDS, Ferris BD, Stevens JP, Lipovsky A, Kazakov T, DiMaio D, Atwood WJ, Sello
662 JK. 2014. Structural optimization of a retrograde trafficking inhibitor that protects cells from
663 infections by human polyoma- and papillomaviruses. *Bioorg Med Chem* 22:4836–4847.
- 664 87. Taube S, Perry JW, Yetming K, Patel SP, Auble H, Shu L, Nawar HF, Lee CH, Connell TD, Shayman
665 JA, Wobus CE. 2009. Ganglioside-linked terminal sialic acid moieties on murine macrophages
666 function as attachment receptors for murine noroviruses. *J Virol* 83:4092–4101.
- 667 88. Han L, Tan M, Xia M, Kitova EN, Jiang X, Klassen JS. 2014. Gangliosides are ligands for human
668 noroviruses. *J Am Chem Soc* 136:12631–12637.
- 669 89. Rydell GE, Svensson L, Larson G, Johannes L, Römer W. 2013. Human GII.4 norovirus VLP induces
670 membrane invaginations on giant unilamellar vesicles containing secretor gene dependent α 1,2-
671 fucosylated glycosphingolipids. *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1828:1840–
672 1845.
- 673 90. Martínez MA, López S, Arias CF, Isa P. 2013. Gangliosides have a functional role during rotavirus
674 cell entry. *J Virol* 87:1115–1122.
- 675 91. Arias CF, Silva-Ayala D, López S. 2015. Rotavirus entry: a deep journey into the cell with several
676 exits. *J Virol* 89:890–893.
- 677 92. Abend JR, Low JA, Imperiale MJ. 2007. Inhibitory effect of gamma interferon on BK virus gene
678 expression and replication. *J Virol* 81:272–279.
- 679 93. Harlow E, Whyte P, Franza BR, Schley C. 1986. Association of adenovirus early-region 1A proteins
680 with cellular polypeptides. *Mol Cell Biol* 6:1579–1589.

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684 **Figure Legends**

685 Figure 1. Visualization of whole genome RNAi screen results. The effects of each siRNA pool are
686 normalized to the non-targeting siRNA control (set as 0) and siTAg (set as 100). Index values from the
687 replicates are illustrated in pairwise scatter plots.

688 Figure 2. Rab18, syntaxin 18, and the NRZ complex are required for BKPyV infection. RPTE cells were
689 transfected with the indicated siRNAs and then infected with BKPyV. Viral infection (TAg), GAPDH or β -
690 actin expression levels, and knockdown efficiency were examined by Western blot.

691 Figure 3. Colocalization of Rab18 and BKPyV capsid protein VP1. RPTE cells were fixed at 6 and 8 hours
692 post infection and were stained for VP1 (green), Rab18 (red), and DAPI (blue). Images were taken using
693 confocal microscopy. Sequential z-stack of images (0.25 μ m increments) are illustrated from the bottom
694 of the cell to the top (left to right). White arrows point to VP1-Rab18 colocalization sites. Bars represent
695 5 μ m.

696 Figure 4. Effects of Rab18 knockdown on BKPyV intracellular trafficking. (A) Rab18, syntaxin 18 (STX18),
697 and NRZ knockdown prevent ER delivery and BKV capsid rearrangement. RPTE cells were transfected
698 with the indicated siRNAs. After 48 hours, they were infected with BKPyV and lysed immediately after
699 adsorption or at 24 hours post-infection under reducing or non-reducing conditions. BKPyV capsid
700 protein VP1, GAPDH, Rab18, STX18, RINT1, and ZW10 levels were examined by Western blot. (B)
701 Alteration of BKPyV intracellular trafficking. RPTE cells were transfected with NTC or siRAB18. BKPyV
702 particles were visualized by immunofluorescent staining for VP1 (green) at 24 hours post infection. Bars
703 represent 100 μ m.

704 Figure 5. Colocalization of BKPyV and organelle markers. RPTE cells were transfected with NTC or
705 siRAB18, then stained for VP1 (green) and the indicated organelle markers (above each row of panels,
706 red) at 24 hours post infection. Images were taken with an inverted fluorescent microscope. Bars
707 represent 100 μ m.

708 Figure 6. Colocalization of VP1 and Rab7. RPTE cells were transfected with NTC or siRAB18, then stained
709 for VP1 (green) and Rab7 (red) at 24 hours post infection. Images were taken with a confocal
710 microscope. Bars represent 10 μ m.

711 Figure 7. Model of BKPyV vesicular trafficking. (A) BKPyV enters a vesicle from the membrane of the
712 Golgi apparatus or the endosome. (B) GTP-bound Rab18 interacts with ZW10 of the NRZ tethering

713 complex. (C) Syntaxin 18 on the ER membrane interacts with v-SNARE. (D) Syntaxin 18 and v-SNARE
714 mediate vesicle fusion.

Figure 1

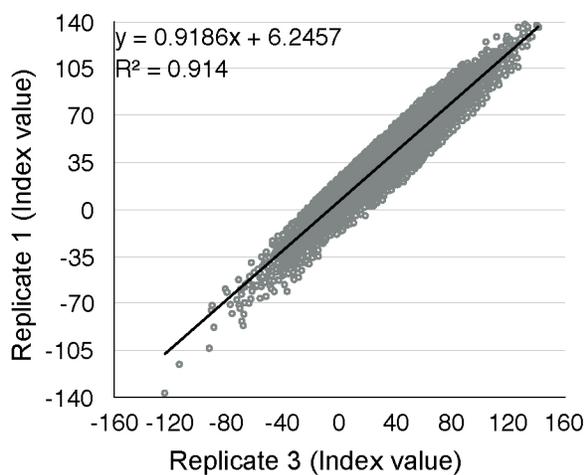
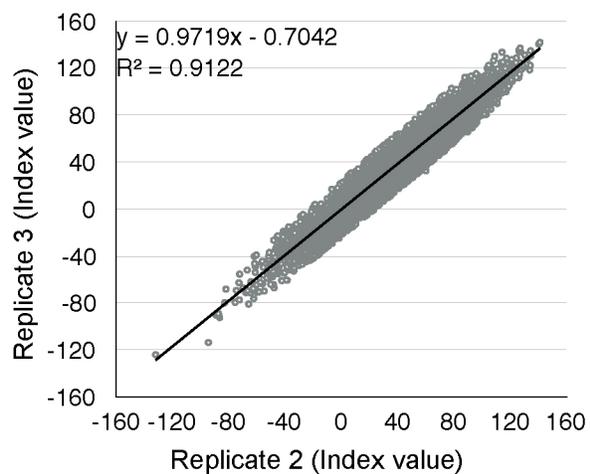
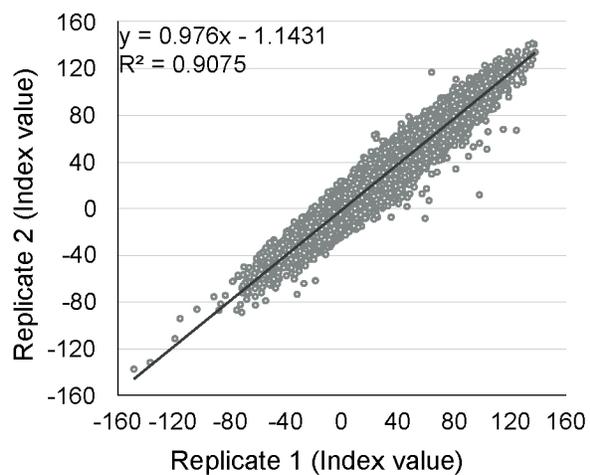
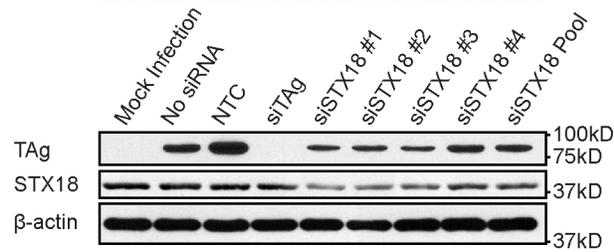
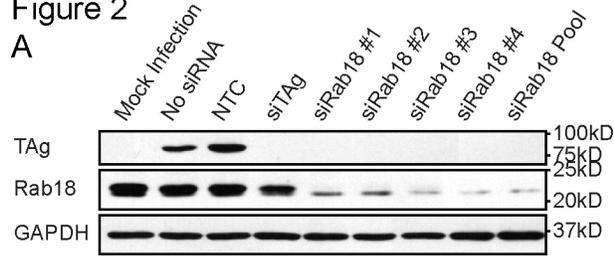


Figure 2

A



B

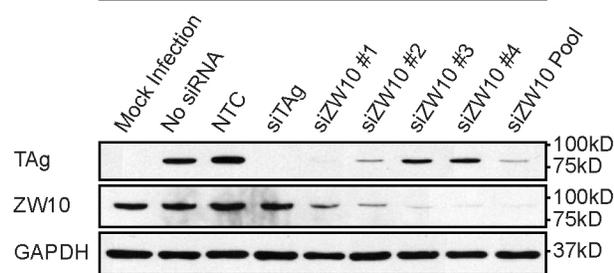
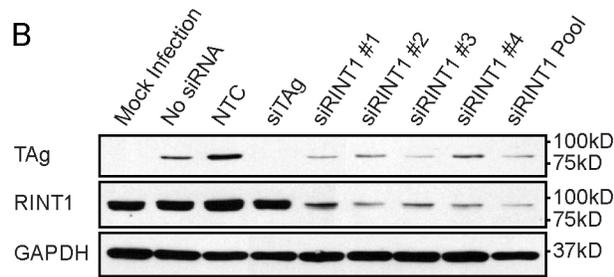


Figure 3

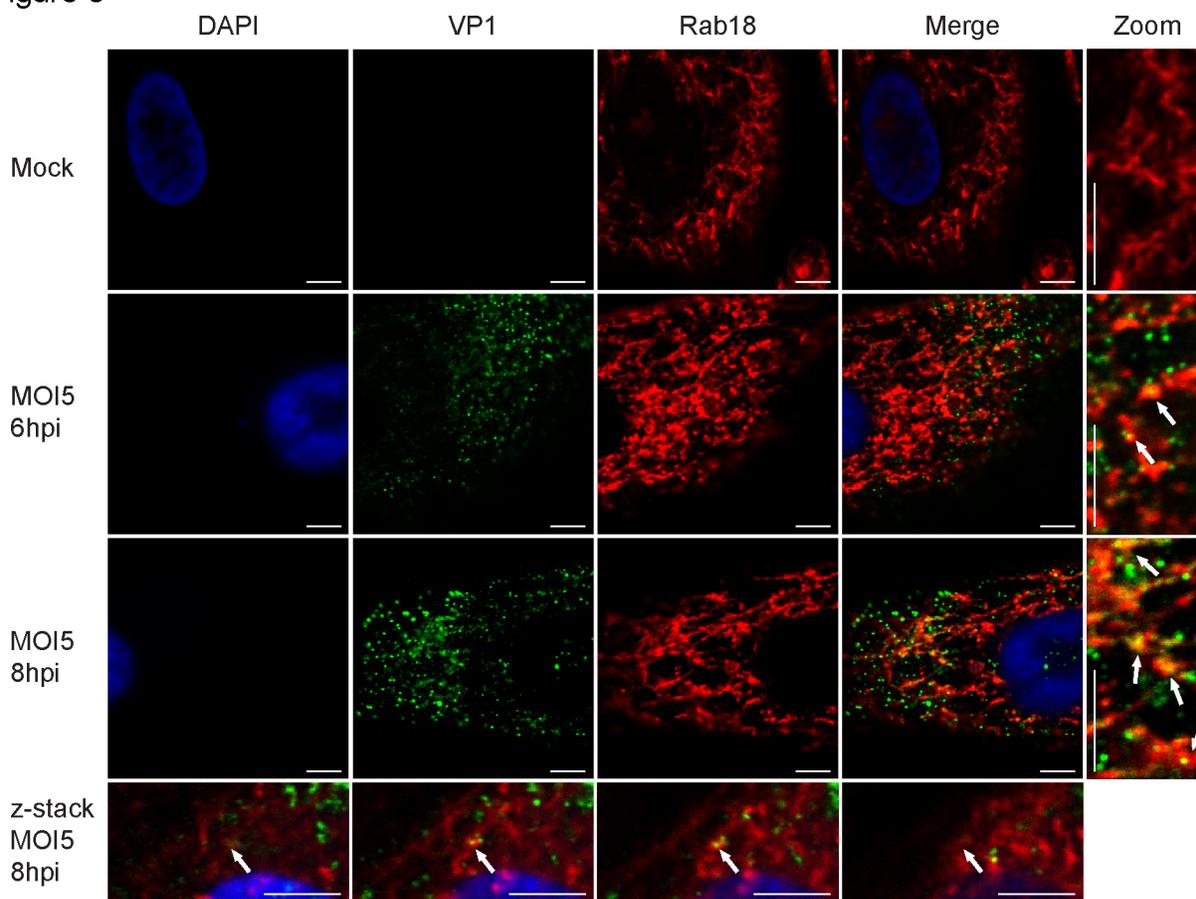
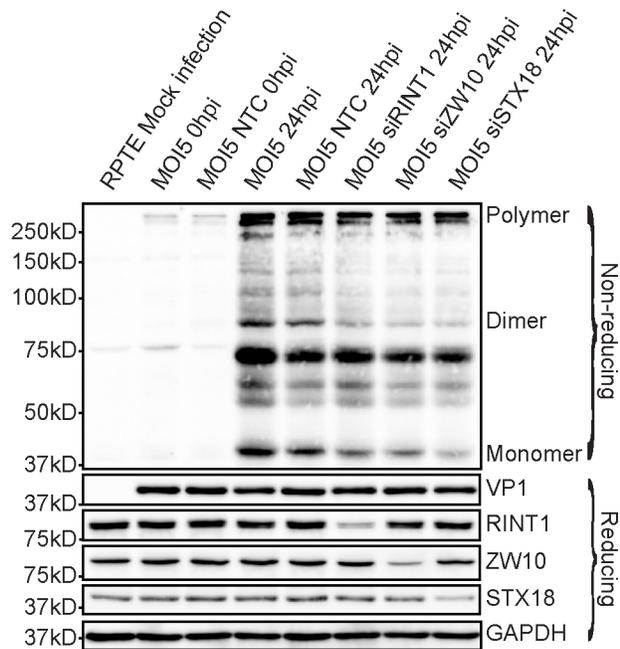
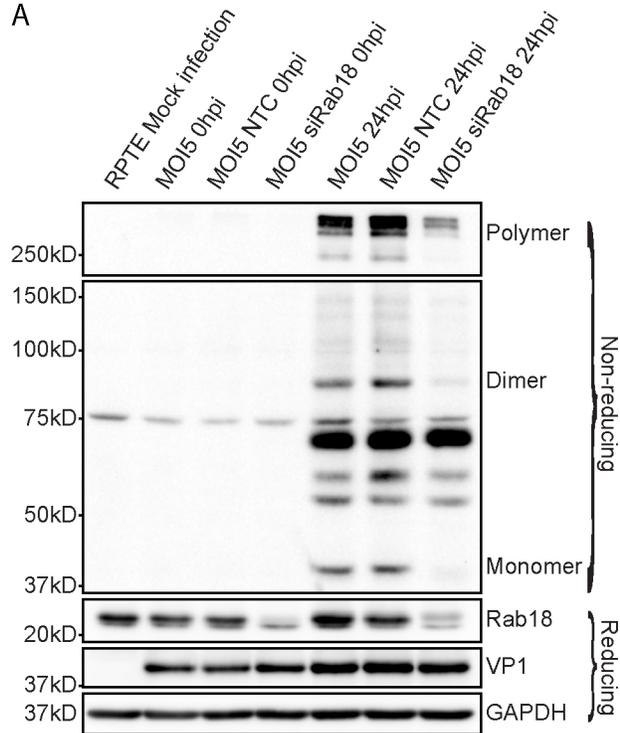


Figure 4

A



B

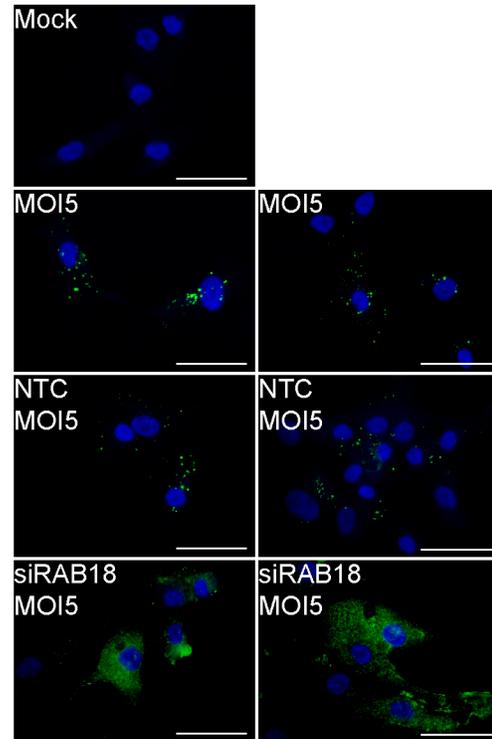


Figure 5

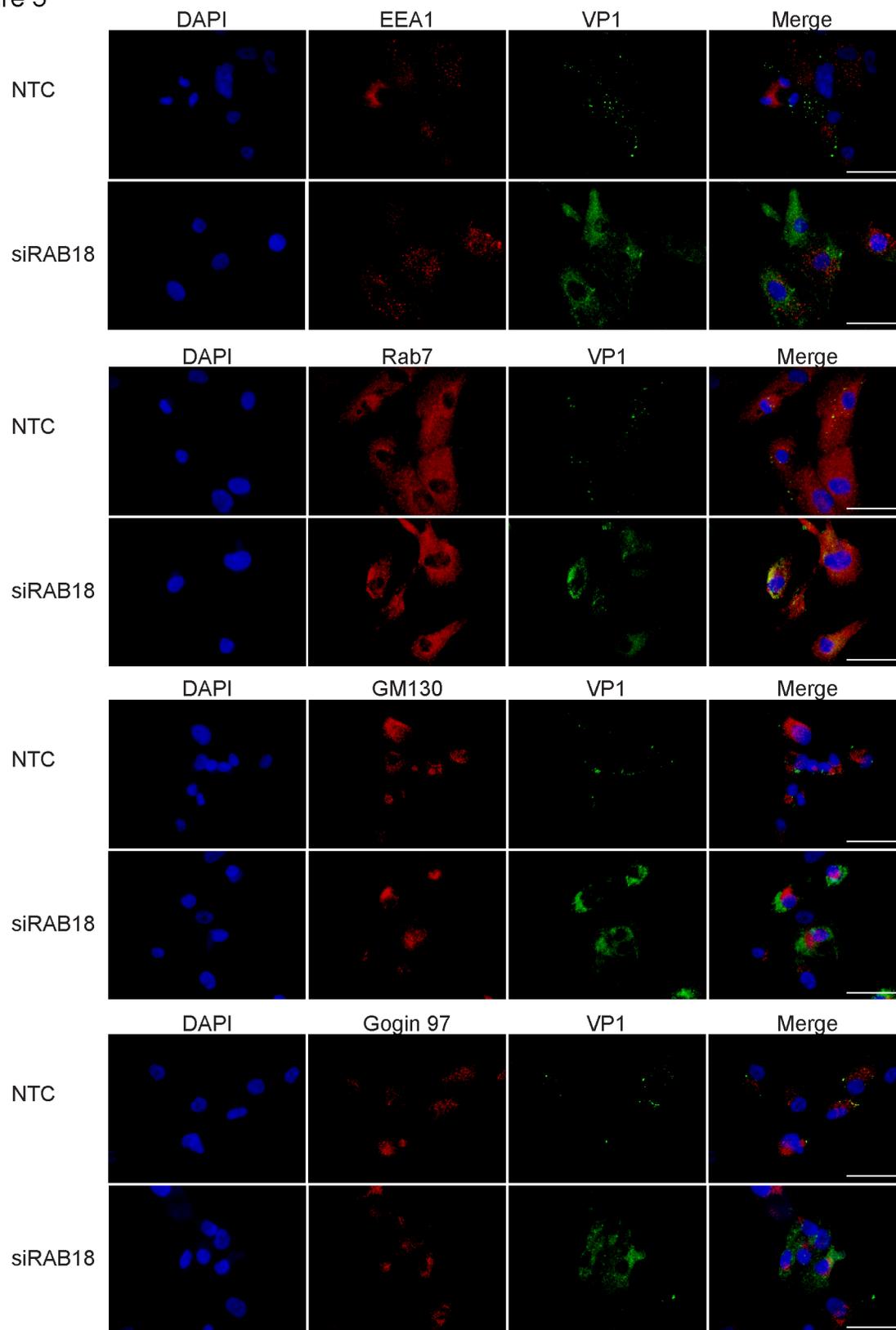


Figure 6

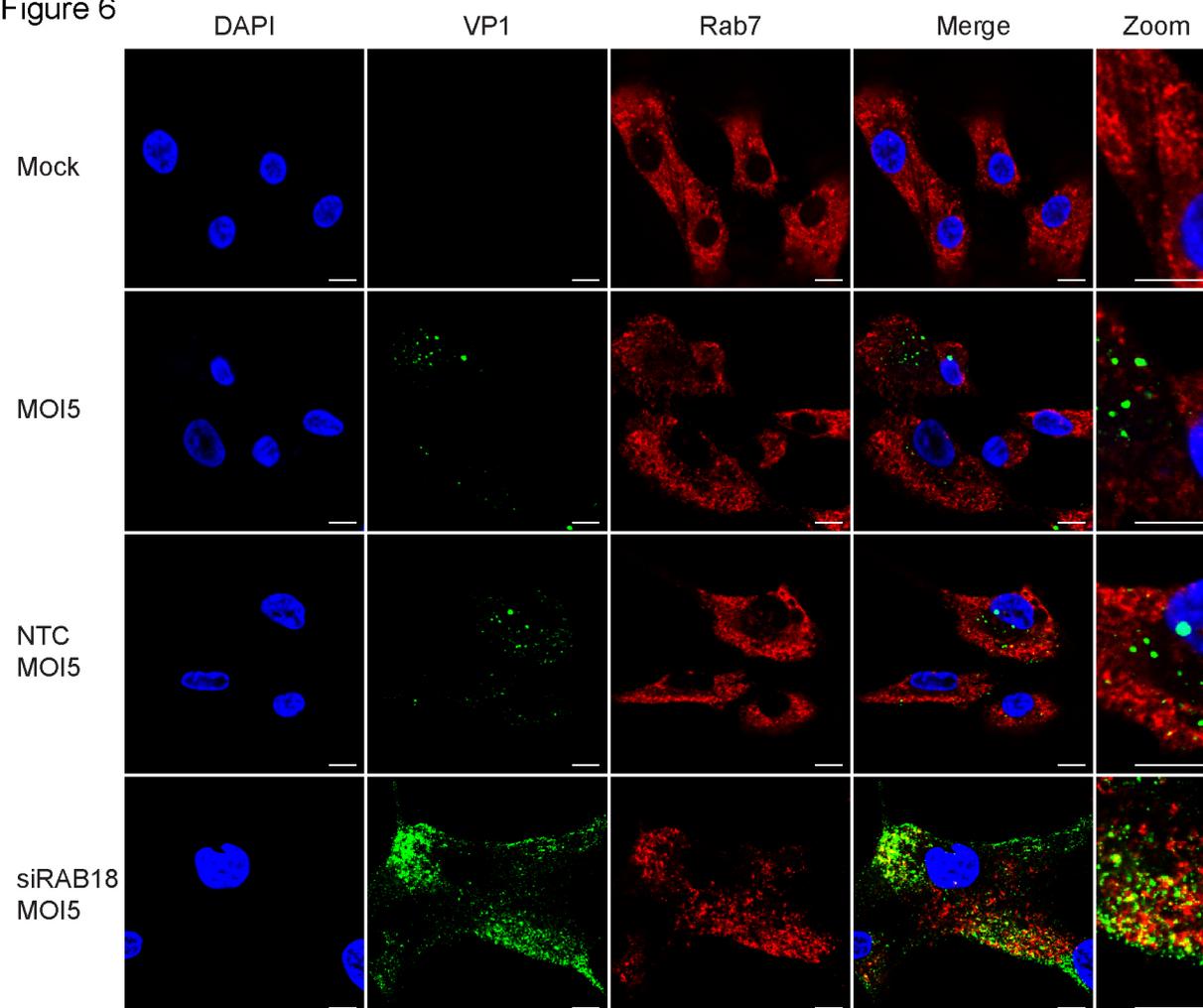


Figure 7

