

1 **Infectious Entry of Merkel Cell Polyomavirus**

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Running title: MCPyV entry into host cells

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22 **endocytosis**

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27 **Abstract**

28 Merkel Cell Polyomavirus (MCPyV) is a small, non-enveloped tumor virus associated
29 with an aggressive form of skin cancer, the Merkel cell carcinoma (MCC). MCPyV
30 infections are highly prevalent in the human population with MCPyV virions being
31 continuously shed from human skin. However, the precise host cell tropism(s) of
32 MCPyV remains unclear: MCPyV is able to replicate within a subset of dermal
33 fibroblasts, but MCPyV DNA has also been detected in a variety of other tissues.
34 However, MCPyV appears different from other polyomaviruses as it requires sulfated
35 polysaccharides such as heparan sulfates and/or chondroitin sulfates for initial
36 attachment. Like other polyomaviruses, MCPyV engages sialic acid as a (co-
37)receptor. To explore the infectious entry process of MCPyV, we analyzed the cell
38 biological determinants of MCPyV entry into A549 cells, a highly transducible lung
39 carcinoma cell line, in comparison to well-studied simian virus 40 and a number of
40 other viruses. Our results indicate that MCPyV enters cells via caveolar/lipid raft-
41 mediated endocytosis but not macropinocytosis, clathrin-mediated endocytosis or
42 glycosphingolipid-enriched carriers. The viruses internalized in small endocytic pits
43 that led the virus to endosomes and from there to the endoplasmic reticulum (ER).
44 Similar to other polyomaviruses, trafficking required microtubular transport,
45 acidification of endosomes, and a functional redox environment. To our surprise, the
46 virus was found to acquire a membrane envelope within endosomes, a phenomenon
47 not reported for other viruses. Only minor amounts of viruses reached the ER, while
48 the majority was retained in endosomal compartments suggesting that endosome-to-
49 ER trafficking is a bottleneck during infectious entry.

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53 **Importance**

54 MCPyV is the first polyomavirus directly implicated in the development of an
55 aggressive human cancer, the Merkel Cell Carcinoma (MCC). Although MCPyV is
56 constantly shed from healthy skin, MCC incidence increases among aging and
57 immunocompromised individuals. To date, the events connecting initial MCPyV
58 infection and subsequent transformation still remain elusive. MCPyV differs from
59 other known polyomaviruses concerning its cell tropism, entry receptor requirements,
60 and infection kinetics. In this study, we examined the cellular requirements for
61 endocytic entry as well as the subcellular localization of incoming virus particles. A
62 thorough understanding of the determinants of the infectious entry pathway and the
63 specific biological niche will benefit prevention of virus-derived cancers such as
64 MCC.

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79 **Introduction**

80 Polyomaviruses (PyV) are small, non-enveloped dsDNA viruses with a diameter of
81 45-50 nm. The icosahedral (T=7) capsids consist of 72 homopentameric capsomers of
82 the major capsid protein VP1 with minor capsid proteins VP2/VP3 located within a
83 cavity underneath the VP1 pentamers. The PyV capsid harbors a chromatinized,
84 circular dsDNA genome of about 5 kb (1, 2). Well-studied PyV such as simian virus
85 40 (SV40), and murine PyV (mPyV) possess a broad cell tropism and can transform
86 cells *in vitro* and in animals (3, 4). Of the human polyomaviruses, JC and BK virus
87 are the best studied (5, 6). JC and BK viruses were initially identified in brain and
88 urine samples, respectively (7, 8). Initial infection with these viruses occurs early in
89 life and typically leads to persistent infections that are typically benign (9-11). Upon
90 immunosuppression, however, persistent JC and BK virus infections may lead to
91 severe diseases, such as progressive multifocal leukoencephalopathy or PyV-
92 associated nephropathy, with potentially fatal outcomes (3, 4).

93

94 In 2008, Feng and colleagues identified Merkel cell polyomavirus (MCPyV) in a rare
95 form of skin cancer known as Merkel cell carcinoma (MCC) (12). MCC is an
96 aggressive cancer with increasing incidence (13, 14), which is most likely to develop
97 in immunocompromised and elderly populations upon prolonged UV exposure (15,
98 16). About 80% of MCC are positive for MCPyV DNA integrated into the host
99 genome (12). As for most PyV, MCPyV infections are widespread and predominantly
100 asymptomatic. In fact, MCPyV is continuously shed from healthy skin with a
101 prevalence of 60-80% (17-19). However, MCPyV DNA has also been isolated from
102 respiratory, urine and blood samples (20), and the range of tissues in which
103 persistent infection can be established is thus still unclear. The presence of
104 integrated MCPyV DNA in MCC cells is thought to cause cancer through the

105 continuous expression of the transforming large T (LT) and small T (sT) antigens.
106 Integration of the viral DNA into the host cell genome is coupled to truncation of the
107 LT C-terminal domain, which is important for viral genome replication and can induce
108 p53 activity, triggering cell cycle arrest (21, 22). The viability of MCC cells depends
109 on expression of LT and/or sT, as pan-T knock-down in MCC-derived cells lead to
110 cell death (23, 24). Importantly, the cellular origin of MCC is still under debate. A
111 recent report suggests dermal fibroblasts as target cells for productive infection,
112 whereas Merkel cells are not permissive for virus entry or productive infection (25,
113 26). Thus, it remains unclear exactly which events give rise to MCC.

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115 Since cell culture systems to produce sufficient quantities of infectious MCPyV are
116 not readily available, MCPyV vectors, so called pseudoviruses (PsV), are important
117 tools to study entry. As MCPyV does not contain detectable levels of VP3 (27), PsV
118 consist of VP1/VP2-only capsids that harbor a reporter plasmid (e.g. coding for
119 EGFP or luciferase). Expression of the reporter allows easy readout for successful
120 entry, i.e. delivery of the viral DNA to the site of transcription and replication (28). In
121 an effort to identify MCPyV-permissive cell lines and to better understand the tissue
122 tropism of MCPyV, the tumor cell library NCI-60 was screened for transducibility and
123 ability to support virus replication with MCPyV PsV and native virions, respectively
124 (29). Of those, A549 cells, a non-small cell lung cancer cell line, showed robust
125 transducibility with MCPyV PsV (28).

126

127 Since MCPyV is an emerging virus, little is known about the basic biology of the
128 virus, in particular how initial infection occurs. Initial studies on the mechanism of
129 MCPyV infection addressed cell surface interactions and cellular tropism of MCPyV
130 (25, 28, 30). MCPyV relies on binding sulfated glycosaminoglycans (GAGs) for initial

131 attachment similar to papillomaviruses (28). However, it also requires interaction with
132 carbohydrates containing a linear sialic acid motif, i.e. Neu5Ac α 2-3Gal, like other
133 PyV (28, 30, 31).

134

135 Different viruses utilize distinct preexisting cellular endocytosis pathways for
136 infectious entry (32). These endocytic pathways are characterized by a specific set of
137 cellular factors and regulators facilitating endocytic vesicle formation. This set of
138 cellular factors is used to distinguish different pathways. Well-studied endocytic
139 pathways include clathrin-mediated endocytosis (CME), caveolar/lipid raft-mediated
140 endocytosis, and macropinocytosis (33), which are essential for receptor-mediated
141 endocytosis, turnover of plasma membrane receptors and fluid-phase uptake. To
142 facilitate safe delivery and release of the viral genome to and at the site of replication,
143 viruses are routed through specific intracellular target organelles, such as
144 endosomes, the Golgi apparatus, or the endoplasmic reticulum (ER).

145

146 Host cell entry of most PyV occurs by caveolar/lipid raft-mediated endocytosis (34-
147 37), whereas JC virus, for example, uses CME (38). After endocytosis, virions are
148 routed through the endosomal pathway to be delivered to the ER (39). There, a
149 chaperone- and disulfide isomerase-mediated uncoating step occurs, whereupon the
150 modified particles are translocated into the cytosol by the ER-associated degradation
151 machinery (40-43). In the current study, we aimed to identify the cellular
152 requirements for virus entry into host cells to understand similarities and differences
153 to other PyV. For this, we used small compound inhibitors of decisive cellular
154 factors/processes during MCPyV infection. As functional controls, we employed well-
155 studied viruses, including SV40, the papillomavirus HPV16, or the alphavirus Semliki
156 Forest Virus (SFV) (36, 44, 45). In addition, we characterized the entry route of

157 MCPyV by ultrathin section transmission electron microscopy (TEM). Our results
158 support a model in which MCPyV enters A549 cells by caveolar/lipid-raft dependent
159 endocytosis, and where viruses are routed through the endosomal pathway to the
160 ER. To our surprise, we found that MCPyV acquired a lipid membrane within
161 endosomes. This membrane was absent once the virus located in the ER.

162 **Results**

163 **MCPyV PsV cell binding and infection depend on sulfated glycosaminoglycans**

164

165 To study the infectious entry requirements of MCPyV, we tested the susceptibility of
166 the keratinocyte-derived cell lines HeLa and HaCaT in comparison to A549 cells.
167 Using PsV we found that neither HeLa nor HaCaT cells showed GFP expression
168 72 h post infection and are thus poorly permissive for MCPyV entry (Fig. 1 A). We,
169 therefore, decided to use A459 cells for our study, which allow for infectious
170 internalization as previously described (29).

171

172 As initial strategy to elucidate the cellular route(s) of entry, perturbation of cellular
173 processes followed by infection is key to identify factors/organelles facilitating
174 MCPyV entry. The time course of MCPyV infectious entry into A549 cells was rather
175 slow and plateaued after 72 h post infection (p.i.) (data not shown). Since indirect
176 effects occur often after prolonged cellular perturbation, it is important to minimize
177 such effects (46). For this, we compared siRNA-mediated knockdown, expression of
178 dominant negative mutants, and small compound inhibition (data not shown). The
179 efficacy and reliability of siRNA-mediated knockdown and expression of dominant
180 mutants turned out to be questionable, as judged by infection of control viruses, so
181 that we turned exclusively to small compound inhibition.

182

183 We first tested whether inhibition of acidification of endosomal compartments with the
184 weak base ammonium chloride (NH₄Cl) perturbed MCPyV infection (47). In the
185 presence of NH₄Cl, MCPyV infection was blocked in a dose-dependent fashion (Fig.
186 1 B) similar to infection with SV40 and HPV16, which served as positive controls (45,

187 48). This indicates the requirement for low pH in endosomal compartments during
188 infectious internalization of MCPyV.

189

190 SV40 is directed to the ER for uncoating and subsequent translocation to the cytosol
191 using ER resident disulfide oxidoreductases and the ER-associated degradation
192 machinery (40). Dithiothreitol (DTT) is a powerful reducing agent and thus perturbs
193 the cellular redox environment and disulfide oxidoreductase functions (49).
194 Accordingly, DTT treatment arrests SV40 in the ER (40). We tested whether MCPyV
195 was also sensitive to DTT treatment. MCPyV and SV40 infectivity was clearly
196 reduced in the presence of DTT, whereas entry of Semiliki Forrest virus (SFV), which
197 does not require a redox-driven ER uncoating step (44), was unaffected (Fig. 1 C).

198

199 Next, the ability of NH₄Cl or DTT to block MCPyV infection in relation to the time
200 course of entry was tested. For this, the drugs were added different times p.i.. NH₄Cl
201 and DTT exhibited a similar propensity to block MCPyV infection, where infectivity
202 was inhibited upon early addition and increased with additions at later time point (Fig.
203 1 D). The increase at later time points indicates that the virus had passed the step
204 blocked by the drugs. At 30 h p.i., about 50% of MCPyV had passed the acid-
205 activated step, whereas the redox-dependent step appeared to occur slightly later
206 (Fig. 1 D). The time courses indicate in addition a rather slow and asynchronous
207 entry process akin to HPV16 (Fig. 1 D, (45)).

208

209 Prolonged treatment of cells with drugs that block endocytosis and trafficking can
210 have indirect or cytotoxic effects. To address this problem, we employed an inhibitor
211 swap approach in which cells were transiently treated with a drug of interest followed
212 by replacement by another that blocks entry at a later stage, such as acid activation

213 in endosomes or redox-mediated uncoating in the ER (45, 46). Moreover, several
214 different viruses served as positive and negative controls to assess the efficacy of
215 treatment as well as the potential for pleiotropic drug effects.

216

217 Initially, as proof of feasibility, the requirement for sulfated GAGs was tested. MCPyV
218 attachment to cells generally depends on such GAGs (28, 30, 31). Therefore,
219 treatment with sodium chlorate (NaClO_3) was used to induce production of
220 undersulfated GAGs (50). Treatment of A549 cells with 10, 20, or 30 mM NaClO_3 for
221 16 h prior to inoculation with MCPyV produced a dose-dependent reduction in
222 infectivity (Fig. 1 E). HPV16 infectivity was similarly reduced, as expected (51). In
223 contrast, infection with SV40 was unaffected, suggesting different requirements for
224 the two PyV (Fig. 1 E). In addition, we tested the inhibitory effect of heparin, a highly
225 sulfated GAG, on MCPyV infection. In line with previous results (28), preincubation of
226 MCPyV pseudovirions with heparin efficiently blocked infection, only slightly less
227 efficiently than the positive control HPV16 (Fig. 1 F). Influenza A virus (IAV) was
228 used as a negative control, as IAV infection depends on interaction with sialic acids
229 (52) and is independent of GAG binding. Heparin did not affect IAV infectivity (Fig. 1
230 F). Taken together, the results confirm that MCPyV infection requires interactions
231 with sulfated cell-surface GAGs.

232

233 **Inhibitory profile of MCPyV endocytosis**

234

235 After confirming previous reports, we set out to characterize the endocytic pathway
236 employed by MCPyV. First, CME was blocked with the small molecule inhibitor
237 pitstop2, which interacts with the clathrin N-terminal domain and thereby interferes
238 with assembly of the clathrin coat (53). Infection with MCPyV was unaffected by

239 treatment with pitstop2 (Fig. 2 A), similar to SV40, which enters cells by
240 caveolar/lipid-raft endocytosis (34, 36, 48). As expected, infection with SFV, which
241 enters cells by CME (44, 54), was efficiently reduced. Thus, MCPyV entry occurs
242 independently of CME.

243

244 Another important regulator of several endocytic pathways is dynamin-2 (Dyn2). The
245 large GTPase regulates scission of endocytic pits in several endocytic pathways, i.e.
246 CME, caveolae/lipid raft-mediated endocytosis, IL-2 endocytosis, and phagocytosis
247 (32). To study a potential Dyn2 involvement in MCPyV uptake, dynasore (an inhibitor
248 of the dynamin GTPase activity) was employed (55). SV40 served as a positive
249 control as it is known to depend on Dyn2 activity (56). Infection of MCPyV was
250 blocked dose-dependently by dynasore to a residual level of $22\pm 13\%$, similar to
251 SV40 (Fig. 2 B). As a negative control, HPV16 was used. HPV16 enters host cells by
252 a clathrin-, caveolin-, cholesterol- and dynamin-independent, but actin-dependent
253 endocytic pathway (45). As expected, HPV16 infection remained unperturbed or
254 even increased after dynasore treatment (Fig. 2 B). This indicates that MCPyV is
255 endocytosed by a Dyn2-dependent pathway similar to SV40 but distinct from HPV16
256 endocytosis.

257

258 To further ascertain that MCPyV enters cells by a similar endocytic mechanism as
259 SV40, we perturbed cholesterol-dependent caveolar/lipid-raft endocytosis. To this
260 end, we treated cells with nystatin and progesterone, which sequester cholesterol
261 and prevent cholesterol synthesis, respectively (34, 56-59). As expected, infection
262 with MCPyV and SV40 were inhibited upon perturbation of cholesterol to residual
263 levels of $31\pm 8\%$ and $34\pm 11\%$, respectively (Fig. 2 C), whereas HPV16 infection

264 remained unaffected as described previously (45). This suggests that MCPyV
265 infection, like SV40 infection, requires cholesterol-rich membrane domains (34).

266

267 Next, a potential role for macropinocytosis was assessed. The Na⁺/H⁺-exchanger
268 regulates macropinocytosis by controlling submembraneous pH, which in turn
269 regulates the formation of membrane protrusions (60). Ethylisopropylamiloride
270 (EIPA)-mediated inhibition of the Na⁺/H⁺-exchanger (61) is a classical treatment to
271 interfere with macropinocytosis. This treatment neither affected MCPyV nor SV40
272 infection (Fig. 2 D), whereas HPV16 infection was efficiently blocked as expected
273 (45). Thus, MCPyV endocytosis occurs independently of the Na⁺/H⁺-exchanger and,
274 consequently, also of macropinocytosis or related pathways.

275

276 In summary, MCPyV entry required cholesterol-rich membranes and dynamin, but
277 did not require clathrin or macropinocytic pathways. These observations are most
278 consistent with an entry pathway that employs caveolar/lipid-raft mediated
279 endocytosis.

280

281 **Regulation of MCPyV endocytosis depends on actin dynamics, Rho-like**
282 **GTPases and signaling via Tyr kinases and phosphatases PP1 and PP2A/B**

283

284 Next, we addressed functional regulators involved in endocytic processes with
285 respect to their role in MCPyV entry.

286

287 Actin polymerization is required for most endocytic pathways employed by viruses,
288 where formation of protrusions, intracellular transport or vesicle scission is facilitated
289 (32, 45). Cytochalasin D and jasplakinolide inhibit actin dynamics by either blocking

290 polymerization or depolymerization, respectively (62, 63). Both cytochalasin D and
291 jasplakinolide efficiently blocked MCPyV and SV40 infection (Fig. 3 A, B), where the
292 inhibitory effect of jasplakinolide was less pronounced for SV40. In contrast, CME-
293 mediated uptake of SFV was unaffected by stabilization of actin filaments (Fig. 3 B)
294 and increased upon actin depolymerization (Fig. 3 A).

295
296 Actin polymerization is most often regulated by the activity of pathway-specific Rho-
297 like GTPases (32, 64, 65). Toxin B from *C. difficile* is a broad inhibitor of all Rho-like
298 GTPases (66) and was hence used to assess the relevance of Rho GTPases for
299 infection by MCPyV and SV40. As a negative control, we used HPV16 infection,
300 which was not perturbed by treatment with toxin B, as expected (Fig. 3 C, (45)).
301 Toxin B reduced MCPyV and SV40 infection to $15\pm 18\%$ and to $0.1\pm 0.1\%$ residual
302 infection, respectively (Fig. 3 C). From this we conclude that Rho-like GTPases likely
303 mediate actin-dependent steps during MCPyV endocytosis (Fig. 3 C). Analogous to
304 SV40, the key step may be actin-dependent closure and fission of endocytic vesicles
305 from the plasma membrane (56).

306
307 Ligand-induced activation of tyrosine kinases (tyr-kinases) regulates the activation of
308 several endocytic pathways such as macropinocytosis and caveolar/lipid raft-
309 mediated endocytosis, whereas CME and other pathways are tyr-kinase independent
310 (32, 65). Genistein, a broad and efficient inhibitor of tyr-kinases (67), was used during
311 infection with MCPyV, SV40, and HPV16. As expected for HPV16 and SV40,
312 infection was blocked upon treatment with 200 μM genistein (Fig. 3 D). Interestingly,
313 MCPyV infection was already sensitive to 50 μM genistein treatment (Fig. 3 D)
314 suggesting a strong requirement for tyr-kinases, potentially at multiple levels.

315

316 Next, cellular phosphatases were inhibited with the broadly active sodium
317 orthovanadate, a competitive inhibitor of all phosphatases (68, 69). Entry of MCPyV,
318 SV40 and HPV16 were all strongly inhibited in the presence of orthovanadate (Fig. 3
319 E). Okadaic acid, a more specific inhibitor of phosphatases families PP1, PP2A and
320 PP2B (70), also inhibited MCPyV, SV40 and HPV16 (Fig. 3 F).

321
322 In summary, MCPyV entry is likely facilitated by dynamic actin rearrangement that is
323 regulated by Rho-like GTPases, as well as by the activity of tyr-kinases and PP1
324 and/or PP2A/B phosphatases.

325

326 **Intracellular trafficking of MCPyV requires endosomal acidification, functional**
327 **microtubular dynamics, and an intact redox environment**

328

329 Treatment with NH₄Cl reduced MCPyV infection indicating an acid-activation step
330 (Fig. 1 B). To test whether this acid-activated step occurred in endosomes,
331 bafilomycin A1, an inhibitor of the endosomal proton pump V-ATPase, was used (71).
332 Bafilomycin A1, like NH₄Cl, inhibited the infectivity of MCPyV, SV40, and HPV16 in a
333 dose-dependent fashion (Fig. 4 A). Thus, MCPyV depends on low endosomal pH for
334 either acid activation or trafficking within maturing endosomes.

335

336 Most intracellular transport occurs along microtubules (72). To assess microtubule
337 involvement in MCPyV infection, depolymerization of microtubules by treatment with
338 the polymerization blocker nocodazole was used (73). As reported previously, SV40
339 and HPV16 entry require the integrity of microtubules (Fig. 4 B). Similarly, MCPyV
340 infection strongly depended on intact microtubular dynamics (Fig. 4 B). This suggests

341 that MCPyV requires microtubules for transport of vesicular compartments or of the
342 virus itself during host cell entry.

343

344 To study the involvement of intracellular transport processes from the ER to the Golgi
345 apparatus in MCPyV entry, we used brefeldin A, which eventually leads to Golgi
346 collapse into the ER (74). Upon perturbation with brefeldin A, infection with MCPyV,
347 SV40, and HPV16 were blocked (Fig. 4 C). This indicates that infection with these
348 viruses requires the functional integrity of the secretory ER/Golgi compartments.

349

350 Another hint for an involvement of the ER during infectious internalization can be
351 drawn from the perturbation of the cellular redox environment by DTT. At the
352 concentrations used, DTT interferes mostly with the formation of disulfide bonds
353 during folding in the ER but not with existing disulfide bonds in folded proteins (49).
354 Since MCPyV infection was blocked by DTT (Fig. 1 C), it is reasonable to assume
355 that MCPyV like SV40 requires an ER step for host cell entry, possibly for uncoating
356 and translocation into the cytosol.

357

358 After escape from the ER, SV40 is thought to be imported into the nucleus via the
359 nuclear pore complexes (75). In contrast, HPV16 requires cell cycle progression, i.e.
360 nuclear envelope breakdown, for nuclear entry (76, 77). To address whether MCPyV
361 infection depends on mitotic activity for nuclear entry, we blocked cells in S-phase
362 using aphidicolin, an inhibitor of DNA polymerase alpha and delta (78). MCPyV entry
363 was efficiently blocked by aphidicolin to a similar extent as HPV16 (Fig. 4 D). The
364 RNA virus SFV replicates in the cytoplasm and therefore served as a negative
365 control (79). SFV infection was not affected by aphidicolin treatment (Fig. 4 D), as

366 expected. These results indicate that MCPyV infection may depend on the mitotic
367 activity of the host cells.

368

369 **MCPyV is internalized into small, non-coated vesicles**

370

371 Our inhibition studies indicate that MCPyV and SV40 use similar pathways to infect
372 cells. To confirm this notion, we examined virion trafficking using TEM. Localization
373 of virus particles was assessed 2, 8, 16, 24 and 48 h p.i.. Interestingly, the virus
374 localized to several specific cellular compartments at each time-point, in line with
375 asynchronous internalization and trafficking of the MCPyV particles. Virus particles
376 were readily detectable bound to the cell surface (Fig. 5 A). In addition, they were
377 found within small, inward budding membrane invagination without any visible protein
378 coat (Fig. 5 A, B). Interestingly, virions resided in two distinct but equally frequent
379 populations of invaginations. In one class of invagination, the plasma membrane
380 appeared to make close contact with the virion surface, while the second class of
381 invagination exhibited $10 \text{ nm} \pm 8 \text{ nm}$ distances between the membrane and the virion
382 surface. The first class of invagination is reminiscent of prior observations for SV40,
383 which is known to make close contact with the sialylated headgroups of plasma
384 membrane glycolipids (80, 81). The latter class of invagination suggests possible
385 interactions between the virion and a glycoprotein receptor with a large ectodomain,
386 such as an HSPG. In addition, MCPyV virions were found within intracellular vesicles
387 without an apparent protein coat (Fig. 5 C). These data, and the absence of virus in
388 large, coated or tubular invaginations, is consistent with MCPyV uptake by
389 caveolar/lipid raft-mediated endocytosis rather than uptake by macropinocytosis,
390 CME or glycosphingolipid-enriched carriers (58, 81-83).

391

392 **MCPyV traffics via the endolysosomal route to the ER**

393

394 After initial uptake into endocytic vesicles, MCPyV particles were found in early
395 endosomal compartments and later accumulated in late endosomal/lysosomal
396 structures (Fig. 6 A, B). Other polyomaviruses, such as SV40, JCPyV and BKPyV,
397 are then trafficked to the ER via Golgi- and non-Golgi routes. In the ER,
398 oxidoreductases and chaperones mediate partial disassembly of the virions
399 (uncoating) and transfer of partially disassembled virions into the cytosol (40, 81, 84-
400 86). MCPyV was undetectable in the Golgi apparatus (Fig. 6 D), the cytosol, or the
401 nucleus. Similar to other PyV, MCPyV was observed in the lumen of the ER, but only
402 to a minor extent (Fig. 6 C).

403

404 Taken together, the EM results thus support the conclusion that MCPyV and SV40
405 use similar infectious entry pathways.

406

407 **MCPyV acquires a lipid envelope in endosomal compartments**

408

409 In addition to the observations described above, we surprisingly found MCPyV
410 particles with what appeared to be a double layer lipid membrane in endosomal
411 compartments (Fig. 7 A, B). The membrane tightly enveloped the particles. The
412 enveloped virions were easily detectable in maturing and late endosomes as well as
413 endolysosomes similar to non-enveloped particles. In fact, both enveloped and non-
414 enveloped particles were detectable side by side in the same endosomes (Fig. 7 C,
415 enveloped black arrows vs. non-enveloped white arrow). This suggested that the
416 process of acquiring a lipid envelope occurs during viral passage of the endosomal
417 pathway rather than in distinct, specialized organelles. It is unclear whether the

418 enveloped particles are part of the productive infectious entry pathway or are instead
419 a dead end for the virus. Possibly, this phenotype reflects a new mode of antiviral
420 defense or a mechanism to evade endosomal degradation.

421

422 **The role of sialic acid for MCPyV entry**

423

424 Previous studies showed that mutations in the sialic acid binding pocket of MCPyV
425 VP1 rendered the particle non-infectious but did not perturb binding to cells (30).
426 Hence, sialic acid interaction is essential for infectious entry, whereas initial
427 attachment occurs mainly by GAG engagement. Since we observed MCPyV particles
428 in endocytic pits in two distinct populations that may reflect binding to gangliosides
429 and HSPGs, two sialic acid binding site mutants (W76A and Y81V) were followed
430 using EM. The mutant PsV assembled without visible defects (Fig. 8 A, B i, C i), but
431 were unable to mediate infection in A549 cells (Fig. 8 D), as expected (30). To verify
432 the desired glycan binding abilities of the sialic acid mutants, we conducted
433 saturation transfer difference (STD-) NMR experiments to probe for binding of
434 sialylated oligosaccharides and GAG oligosaccharides to MCPyV wild-type and
435 mutant virus-like particles (VLPs). STD-NMR makes use of energy transfer from
436 proteins to their ligands upon binding and thus allows determination of binding
437 specificities by comparing the changes in the resonance frequencies of the ligand
438 molecules in association with wild-type or mutant viruses (30, 87). Here,
439 3'-sialyllactose (3'SL) and a chemically well-defined heparan sulfate (HS)-
440 pentasaccharide, Arixtra (Ax), were chosen as a minimal sialylated MCPyV ligand
441 and a short GAG oligosaccharide, respectively (Fig. 9 A, B). STD-NMR showed that
442 the GAG pentasaccharide bound to wild-type VLPs as well as to the W76A mutant
443 (Fig. 9 B ii vs. iii). In contrast, 3'SL bound both wild-type and Y81V mutant VLPs but

444 no saturation transfer was observed from the W76A mutant to 3'SL (Fig. 9 A ii vs. iii
445 vs. iv). Consistent with previous crystallographic and NMR-based results (30), only
446 resonances from the 3'SL sialic acid and galactose rings were observed. For the HS
447 pentasaccharide (Fig. 9 B), on the other hand, magnetization transfer was observed
448 almost equally for all five monosaccharide units, with the non-sulfated GlcA ring
449 being the only unit, whose resonances were somewhat underrepresented in the
450 STD-NMR spectra in comparison to the ^1H reference spectrum of the free
451 pentasaccharide. This suggests that the GlcA ring is the least important determinant
452 in the interaction. In summary, these results confirm that the W76A mutant is
453 defective in sialic acid binding, whereas Y81V remained partially able to interact with
454 the 3'SL ligand under these conditions. Furthermore, the binding site for GAGs
455 apparently remained intact upon mutation suggesting that the GAG binding site is
456 spatially distinct from the sialic acid interaction site.

457

458 EM analyses showed that the W76A and Y81V mutants readily bound to the A549
459 cells, presumably through interactions with HSPGs (Fig. 8 B ii, C ii; (30)).
460 Interestingly, both mutants were exclusively observed in the second class of
461 invaginations (i.e., invaginations with a >5 nm gap between the plasma membrane
462 and the virion surface (Fig. 8 B ii, C ii). This observation supports the concept that the
463 first class of invaginations are formed through short range contacts between the
464 virion and sialylated glycolipids (which the mutants fail to bind), while the second
465 class of invagination involves interactions with bulkier HSPGs.

466

467

468

469

470 **Discussion**

471

472 MCPyV is the first human PyV clearly linked to the development of a specific cancer
473 (12). Initial studies on the principal mechanism of infection confirmed the requirement
474 of sulfated glycosaminoglycans for attachment and sialylated glycans for infectious
475 uptake into host cells, which may, in fact, reflect a common mechanism for several
476 PyV (28, 30, 31, 88). To extend our knowledge of the mechanism of initial infection,
477 we addressed additional cellular requirements and routes of virus entry. Our
478 evidence from inhibitor and morphological studies indicates that MCPyV infects cells
479 asynchronously via a caveolar/lipid-raft dependent endocytic pathway that is similar
480 to that used by SV40. After internalization, virus particles were routed to
481 endolysosomal compartments and the ER. Our preliminary evidence suggests that
482 entry of MCPyV depends on progression of the cell cycle, potentially for the nuclear
483 entry step.

484

485 The initial step of virus entry is binding to target cells. Previous work suggested that
486 the interaction with sulfated GAGs is required for MCPyV binding, whereas sialic
487 acids have a post attachment role (28). We confirmed that heparin, a highly sulfated
488 GAG, was able to compete for cell surface GAGs thus prohibiting binding and
489 infection of A549 cells. In addition, we addressed the role of GAGs and sialic acids
490 concerning their influence on endocytic uptake. Interestingly, we observed two
491 populations of MCPyV particles on the plasma membrane and in endocytic pits
492 differing in their distance to the limiting membrane, possibly representing the different
493 binding receptor species. In principle, these could reflect binding to proteinaceous
494 receptors with a large ectodomain (wide pits) or gangliosides (sialylated lipids, tight
495 pits), as shown for JC virus uptake via sialylated glycoproteins (89) and SV40 uptake

496 by ganglioside interaction (90). Alternatively, the proteinaceous receptor may
497 constitute HSPGs.

498

499 To further unravel the individual roles of sialylated glycans and GAGs during MCPyV
500 entry, two sialic acid binding site mutants (W76A and Y81V, (30)) were subjected to a
501 STD-NMR-based binding assay. These studies confirmed that binding to sialylated
502 glycans was abolished for W76A, but not for Y81V, whereas either mutant retained
503 binding of the HS pentasaccharide. This finding indicates that the binding sites for
504 sialic acid and sulfated GAGs do not overlap and are independent of each other. The
505 most striking difference between wild-type MCPyV and the sialic acid binding site
506 mutants in the TEM experiments was that the mutants were exclusively detected in
507 endocytic pits with the large distances between viral particles and plasma membrane
508 layers. The most likely interpretation of these observations is thus that the large
509 distance reflects binding to a proteinaceous receptor containing sulfated GAGs such
510 as HSPGs, whereas tightly fitted binding to the plasma membrane reflects
511 ganglioside engagement. Since uptake of HSPGs has been suggested to occur
512 primarily through flotillin- and dynamin-dependent endocytosis (91, 92), MCPyV
513 internalization in wider pits via HSPGs seems distinct from the caveolar/lipid raft-
514 mediated internalization required for infection. It thus appears that MCPyV
515 internalization via HSPGs is a dead end. While W76A and Y81V MCPyV were readily
516 observable in endosomes, they were not detected in the ER. Hence, the W76A and
517 Y81V mutations likely rendered the particles non-infectious, as they were unable to
518 engage sialic acid containing gangliosides efficiently and thus were not taken up by
519 an infectious pathway that routes the virus to ER.

520

521 Since the sialic acid binding mutants were found in endosomes but not the ER, the
522 interaction with sialic acid receptors is crucial for routing the virus to the ER. This is in
523 line with previous work indicating that branched sialylated glycosphingolipids targets
524 murine polyomavirus (mPyV) and SV40 from endolysosomes to the ER (36, 48, 80,
525 93). The role of HSPGs remains less clear. It may be that MCPyV interacts with
526 sulfated GAGs simply, because they may serve as an initial high affinity attachment
527 factor, which facilitates later interaction with low affinity glycosphingolipids. An
528 alternative mechanism may be the induction of a conformational change in the virus
529 capsid upon GAG binding that may facilitate uncoating and transfer to the secondary
530 receptor, as it has been described for HPV16, a virus with a similar tissue tropism
531 (51, 94). However, such GAG-induced conformational changes have no precedent
532 among other PyVs. Of note, JC PyV can utilize two distinct pathways for infectious
533 internalization, where one depends on interaction with sulfated GAGs and the other
534 relies on sialic acid binding, this effect could depend on the host cell type (88). As the
535 niche(s) for MCPyV infection remain only partially understood, the role of HSPGs in
536 MCPyV entry may thus depend on specific cell types that are being infected *in vivo*.

537

538 Receptor engagement leads to the endocytosis of MCPyV. In line with our initial
539 hypothesis that infectious entry of MCPyV may follow a path similar to that of SV40,
540 our inhibitory experiments showed that infectious entry was independent of clathrin
541 and the activity Na⁺/H⁺-exchanger but required dynamin and cholesterol, as well as
542 actin dynamics. Since MCPyV particles were not found in coated pits or membrane
543 protrusions, entry does not involve CME or macropinocytosis. The combined
544 requirements for dynamins, cholesterol and actin dynamics indicated endocytosis by
545 caveolar/lipid raft mediated endocytosis. Dynamins are also involved in phagocytosis
546 and endocytosis of interleukin-2 (IL-2), both of which also require actin and lipid

547 rafts/cholesterol (32). Phagocytosis occurs only in specialized cells and it is
548 characterized by long outward protrusions that close around large cargos (95). Thus,
549 an involvement of phagocytosis can be excluded similar to macropinocytosis. IL-2
550 endocytosis, on the other hand, occurs into small, inward budding vesicles, which are
551 mostly formed at the base of an outward protrusion (96). Such initial pits containing
552 MCPyV can be found in the electron micrographs, however in the majority of cases,
553 MCPyV is taken up into small vesicle from flat membrane regions, which makes IL-2-
554 like endocytosis seem unlikely.

555

556 Caveolar/lipid raft-mediated endocytosis routes cargos into endosomal
557 compartments (48, 97). We found MCPyV particles in high abundance in endosomal
558 compartments resembling endosomes, lysosomes, multivesicular and lamellar
559 bodies. We also observed small numbers of MCPyV virions in the ER. Retrograde
560 trafficking from endosomes to the ER is a general mechanism employed by PyVs.
561 The low frequency of ER-localized MCPyV virions is similar to prior observations of
562 mPyV entry (93) and hints at a bottleneck for trafficking from endosomes to the ER.
563 Murine PyV as well as JC and BK PyV have been shown to require acidic pH
564 environments in the endolysosomal compartment presumably for a conformational
565 change facilitating membrane penetration after translocation into the ER by a yet
566 unknown mechanism (86, 98, 99). For MCPyV, acidification in the endolysosomal
567 system may serve a similar role.

568

569 MCPyV infection is sensitive to DTT treatment (Fig. 1 C) and may therefore be
570 dependent on the redox environment within the ER. In analogy to SV40, uncoating
571 and membrane translocation to the cytoplasm may occur in the ER with the help of
572 the ERAD machinery and cytoplasmic chaperones (39, 40).

573

574 As a final step, the viral genome must be delivered to the nucleus, which is the site of
575 early gene expression and replication for PyV. Interestingly, we found that MCPyV
576 infection was sensitive to cell cycle block in S-phase, which indicates that mitosis is
577 required for completion of MCPyV entry. Previous work on HPV16 identified its
578 dependence on mitotic activity of the target cells, which allows delivery of the viral
579 genome to the nucleus upon nuclear envelope breakdown (77, 100). However, SV40
580 has been described to enter the nucleus of interphase cells through the nuclear pore
581 complex by making use of nuclear localisation signals in the viral capsid proteins
582 after ERAD-dependent partial disassembly (40, 101-103). It remains thus unclear,
583 why MCPyV target cells actively progressing through the cell cycle. Nevertheless, our
584 findings are in line with the importance of WNT signalling during infection of dermal
585 fibroblasts (25).

586

587 To our surprise, we regularly observed enveloped particles in endosomal
588 compartments. It remains unclear when and how these virions acquire a
589 membraneous envelope, whether this reflects an antiviral mechanism or whether it is
590 part of the infectious entry mechanisms. It is conceivable that MCPyV uses the
591 ESCRT machinery that generates intraluminal vesicles (ILVs) in multivesicular bodies
592 (104). Alternatively, it may wrap itself in a membrane within the late
593 endosomal/lysosomal compartment. The membrane could theoretically shield the
594 virus from hydrolases, and may give it the time to persist until infection can be
595 completed e.g. by progression through the cell cycle. Alternatively, these enveloped
596 virus particles might arise through an unknown antiviral mechanism. Future studies
597 will address the relevance of such enveloped particles for the infectious route.

598

599 In the light of our experiments and previous reports (28, 30), we propose that MCPyV
600 attachment and internalization, at least in the model A549 cell line, is mediated by
601 HS-type GAGs but that additional sialic acid binding is essential to route the
602 internalized virus to the productive infection pathway of retrograde ER trafficking.
603 However, entry into the ER seems to be a bottleneck for infections.

604

605

606 **Materials and Methods**

607 **Cell lines, antibodies, and reagents**

608 HeLa cells were from ATCC. A549 cells were a kind gift from C. Buck (NIH, National
609 Cancer Institute, Bethesda, MD, USA). CV1 cells were a kind gift from J. Kartenbeck
610 (DKFZ, Heidelberg, Germany). BHK Helsinki cells were a kind gift from A. Helenius
611 (ETH Zürich, Switzerland). 293TT cells were a kind gift from J. Schiller (NIH, National
612 Cancer Institute, Bethesda, MD, USA). Aphidicolin, EIPA, cytochalasin D,
613 nocodazole, nystatin, NH₄Cl, NaClO₃, heparin, and sodium orthovanadate were from
614 Sigma-Aldrich. Bafilomycin A1, genistein, progesterone were from Applichem.
615 Brefeldin A, cyclosporine A, jasplakinolide, okadaic acid were from Calbiochem.
616 Dynasore was from Merck. Pitstop2 was from Abcam. RedDot2 was from VWR.

617

618 **Viruses**

619 MCPyV pseudoviruses (PsV) containing a GFP reporter plasmid (MCPyV-GFP) were
620 produced by transfection of 293TT cells with pwM2m, ph2m and phGluc as described
621 previously (105, 106). In brief, 293TT cells were transfected with the indicated
622 plasmids. After 48 h, cells were harvested and lysed. For optimal maturation, lysates
623 were incubated for further 24 h with 25 mM ammonium sulfate (pH 9.0) (107).
624 MCPyV PsVs were purified on a 25%-39% linear OptiPrep gradient (Sigma-Aldrich).
625 HPV16 pseudoviruses containing a GFP reporter plasmid (HPV16-GFP) were
626 produced by transfection of 293TT cells with p16SheLL and pCIneo as described
627 previously (105, 106). The procedure was similar to MCPyV production above.
628 SV40 and SFV were produced as described previously (40, 108).

629

630 **Infection assays upon inhibitor treatment**

631 Infection assays with MCPyV, HPV16, and SV40 were conducted in 96well plates,
632 where 3000 or 10000 A549 cells were seeded in RPMI (5% FCS and 2 mM
633 glutamine) at least 6 h prior to infection. Cells were treated with 80 μ L of the following
634 inhibitors at the indicated concentrations diluted in RPMI (5% FCS, 2 mM glutamine)
635 or SV40 infection medium (RPMI with 3% BSA, 10 mM HEPES, pH 6.8): overnight
636 preincubation - nystatin/progesterone, sodium chlorate, and toxin B; 30 min
637 preincubation – bafilomycin A1, brefeldin A, cytochalasin D, dithiotreitol, dynasore,
638 EIPA, genistein, jasplakinolide, NH_4Cl , nocodazole, okadaic acid, pitstop2, sodium
639 orthovanadate, wortmannin. Virus was diluted in RPMI (5% FCS, 2 mM glutamine) or
640 SV40 infection medium and 20 μ L of the inoculum were added to each well. MCPyV
641 samples were incubated for 30 h until the inhibitor dilutions were exchanged to RPMI
642 containing 10 mM NH_4Cl and 10 mM HEPES, pH 7.4 and incubated for further 42 h.
643 Samples were fixed by addition of a final 4% paraformaldehyde (PFA) to the wells.
644 Nuclei were stained with RedDot2. Infection scored by analysis of GFP expression by
645 microscopy (Zeiss Axio Observer Z1 equipped with a Yokogawa CSU22 spinning
646 disc module and a CoolSnap HQ camera; Visitron Systems GmbH). Infection with
647 HPV16 was similar to MCPyV but for the exchange of medium to DMEM containing
648 10 mM NH_4Cl and 10 mM HEPES, pH 7.4 occurred after 12 h post infection and
649 fixation was done after total 48 h. SV40 inoculum was replaced by fresh DMEM
650 containing 5 mM DTT after 10 h incubation until fixation after 24 h. Cells were
651 incubated with SFV for 4 h until fixation. Samples were fixed as above. For detection
652 of infection, SV40 and SFV samples were stained with an anti-LTag antibody (sc-
653 20800) or SFV glycoprotein antibody (110), respectively, and an AF488-coupled
654 secondary antibody; subsequently nuclei were stained with RedDot2. Infection was
655 scored by microscopy (Zeiss Axio Observer Z1 equipped with a Yokogawa CSU22
656 spinning disc module and a CoolSnap HQ camera; Visitron Systems GmbH). Cell

657 numbers and infection indices were determined by using the MatLab script Infection
658 Counter as described before (40, 48).

659 Alternatively, the effects of single inhibitors on MCPyV and control virus infection
660 were tested in a flow cytometry-based assay. Here, 3×10^4 A549 cells were seeded in
661 24-well plates or 5×10^4 HeLa cells in 12-well plates or 2.5×10^5 BHK Helsinki cells in
662 12-well plates 6-8 h prior to treatment with aphidicolin over night. The compound was
663 renewed before cells were infected with MCPyV, HPV or SFV, respectively.
664 Aphidicolin was renewed daily until fixation of the cell at 72 h p.i. by trypsinization
665 and subsequent addition of 4% paraformaldehyde. The percentage of GFP-positive
666 cells was measured by flow cytometry using a BD FACScalibur.

667

668 **Electron microscopy**

669 For negative staining EM of virus particles, about 8×10^6 PsV in PBS/0.8M NaCl were
670 absorbed for 5 min on formvar coated, carbon sputtered grids. Particles were
671 contrasted for 7 min with 1% phosphotungstic acid. Samples were analyzed directly
672 after drying. The sample was analyzed at 80 kV on a FEI-Tecnai 12 electron
673 microscope (FEI, Eindhoven, Netherlands). Images of selected areas were
674 documented with Olympus Veleta 4k CCD camera.

675 For ultrathin sectioning transmission EM, virus particles were added for 2 h, 8 h and
676 16 h to A549 cells before fixation with 2.5% glutaraldehyde in PBS. Samples were
677 post-fixed with 0.5% OsO₄, block stained with 0.5% uranyl acetate and after
678 dehydration embedded in Epoxyresin. 60nm ultrathin sections were cut and
679 counterstained with uranyl acetat and lead. Images of selected areas were
680 documented with Olympus Veleta 4k CCD camera.

681

682 **NMR spectroscopy**

683 NMR experiments were conducted at 283 K using a 600 MHz Bruker Avance
684 spectrometer equipped with a TXI triple resonance room temperature probe head. 3
685 mm I.D. NMR tubes with 200 μ L sample volume were used. Each sample contained
686 55.6 nM MCPyV VLPs (i.e. 20 μ M of the major capsid protein VP1) - either wild-type
687 or mutant - and 1 mM oligosaccharide (either 3'SL or the Arixtra GAG-
688 pentasaccharide). Prior to NMR sample preparation, VLPs were dialysed in Slide-A-
689 Lyzer MINI dialysis devices (Thermo Fisher Scientific) against 150 mM NaCl, 1 mM
690 CaCl_2 , pH 6.0 in D_2O . 3'SL (Carbosynth) was added from a 40 mM stock solution
691 prepared in pure D_2O and Arixtra was added from a 7.2 mM stock solution in 150 mM
692 NaCl, 1 mM CaCl_2 , pH 6.0 in D_2O , dialysed from ready-to-inject syringes (Aspen).
693 For 3'SL-containing samples, off- and on-resonance frequencies in STD-NMR
694 experiments were set to -30 ppm and 7.3 ppm, respectively, while -30 ppm and -0.5
695 ppm were used for Arixtra-containing samples, owing to the different chemical shift
696 ranges of both glycans. The irradiation power of the selective pulses was set to 57
697 Hz. The saturation time was 2 s and a total relaxation delay of 3 s was used. A 50-ms
698 continuous-wave spin-lock pulse with a strength of 3.2 kHz was employed to
699 suppress residual protein signals. A total number of 512 scans and a total number of
700 10,000 points were collected and spectra were multiplied with a Gaussian window
701 function prior to Fourier transformation.

702

703 **MCPyV VLP preparation for NMR spectroscopy**

704 MCPyV VLPs were produced essentially according to a published protocol (111). The
705 pwM and ph2m vectors coding for MCPyV VP1 and VP2, respectively, ((27), see also
706 www.addgene.org) were used at a 5:1 ratio for transfection of 293 TT cells. OptiPrep
707 was omitted during the purification and replaced with two CsCl density gradient
708 centrifugation steps.

709

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718 'Virocarb').

719

720 **Figure legends**

721

722 **Figure 1: MCPyV infection is slow and asynchronous, and relies on interaction**
723 **with sulfated glycans.** (A) A549, HeLa or HaCaT cells were infected with increasing
724 amounts of MCPyV PsV. Depicted are relative infection values (%) related to the
725 MCPyV amount yielding 20% absolute infection in A549 cells \pm SD (1x = 80 ng VP1).
726 (B) A549, CV-1 or HeLa cells were infected with MCPyV, SV40 or HPV16,
727 respectively, in the presence of indicated concentrations of NH_4Cl . (C) A549, CV-1 or
728 BHK cells were infected with MCPyV, SV40 or SFV, respectively, in the presence of
729 indicated concentrations of DTT. (D) A549 cells were infected with MCPyV for total
730 72 h, while 10 mM NH_4Cl or 5 mM DTT was added at indicated h p.i.. (E) A549, CV-1
731 or HeLa cells were infected with MCPyV, SV40 or HPV16, respectively, upon
732 pretreatment with indicated concentrations of NaClO_3 for 16 h prior to infection. (F)
733 A549 were infected with MCPyV or IAV, and HeLa cells were infected with HPV16
734 virions, which were treated with indicated concentrations of heparin for 1 h prior to

735 infection. (B-F) Depicted are averages of relative infection values to untreated
736 controls \pm SD from at least 3 independent experiments.

737

738 **Figure 2: MCPyV infection is dynamin- and cholesterol-dependent, but**
739 **independent from clathrin and Na⁺/H⁺-exchangers.** (A) A549, CV-1 or BHK cells
740 were infected with MCPyV, SV40 or SFV, respectively, in the presence of indicated
741 concentrations of pitstop2. (B-D) A549, CV-1 or HeLa cells were infected with
742 MCPyV, SV40 or HPV16, respectively, in the presence of indicated concentrations of
743 dynasore (B), nystatin/progesterone (C), and EIPA (D). Depicted are percentages of
744 infection values relative to solvent treated controls \pm SD of at least 3 independent
745 experiments.

746

747 **Figure 3: MCPyV infection is dependent on actin, RhoGTPases, tyr-kinases,**
748 **and cellular phosphatases.** (A-B) A549, CV-1 or BHK cells were infected with
749 MCPyV, SV40 or SFV, respectively, in the presence of indicated concentrations of
750 cytochalasin D (A) or jasplakinolide (B). (C-F) A549, CV-1 or HeLa cells were
751 infected with MCPyV, SV40 or HPV16, respectively, in the presence of indicated
752 concentrations of toxin B (C), genistein (D), sodium orthovanadate (E), or okadaic
753 acid (F). Depicted are percentages of infection values relative to solvent treated
754 controls \pm SD of at least 3 independent experiments.

755

756 **Figure 4: MCPyV infection requires endosomal acidification, functional**
757 **microtubular dynamics, and an intact redox environment.** (A-C) A549, CV-1 or
758 HeLa cells were infected with MCPyV, SV40 or HPV16, respectively, in the presence
759 of indicated concentrations of bafilomycin A (A), nocodazol (B), and brefeldin A (C).
760 A549, BHK, or HeLa cells were infected with MCPyV, SFV or HPV16 respectively, in

761 the presence of indicated concentrations of aphidicolin (D). Depicted are percentages
762 of infection values relative to solvent treated controls \pm SD of at least 3 independent
763 experiments.

764

765 **Figure 5: MCPyV is taken up into tight-fitting inward budding pits.** (A-C) A549
766 cells were infected with MCPyV particles for 2-24 h before fixation with
767 glutaraldehyde. Cells were processed for TEM according to standard procedures.
768 Images of early entry events were taken. Scale bar 100 nm.

769

770 **Figure 6: MCPyV travels through the endolysosomal system to the ER omitting**
771 **the Golgi.** (A-D) A549 cells were infected with MCPyV particles for 2-24 h before
772 fixation with glutaraldehyde. Cells were processed for TEM according to standard
773 procedures. Virus particles were found in endosomal compartments (A, B) and in the
774 ER (C). MCPyV was absent from the Golgi (D). Scale bar 100 nm.

775

776 **Figure 7: MCPyV virions acquire a membrane envelope during cell entry.** (A-C)
777 A549 cells were infected with wild-type MCPyV particles before fixation with
778 glutaraldehyde. Cells were processed for TEM according to standard procedures.
779 Images of virus particles in intracellular compartments were taken after 8 h and 16 h
780 p.i.. Note the envelope around the virus particles in the representative endosomal
781 compartments. C) Note that enveloped particles (black arrows) are found side-by-
782 side with non-enveloped virions (white arrow) in the same organelle. Scale bars 100
783 nm; Scale bar inset 50 nm.

784

785 **Figure 8: MCPyV sialic acid binding mutants are taken up into A549 cells but**
786 **fail to mediate infection.** (A-C i) MCPyV wild-type and mutant particles were

787 analyzed by electron microscopy after negative staining. Depicted are representative
788 images of virions. Scale bars: 50 nm. (B-C ii and iii) A549 cells were infected with
789 W76A and Y81V MCPyV particles for 2-24 h before fixation with glutaraldehyde.
790 Cells were processed for TEM according to standard procedures. Images of virus
791 particles in endocytic pits and in intracellular compartments were taken after 8 h (ii)
792 and 16 h p.i. (iii), respectively. Scale bar 100 nm for ii and 200 nm for iii. (D) 60 ng
793 (1x), 300 ng (5x) and 600 ng (10x) of MCPyV wild-type and W76A and Y81V mutant
794 particles were used in an infection assay with A549 cells for 72 h.

795

796 **Figure 9: MCPyV sialic acid binding mutants are capable of GAG binding.** A)
797 STD-NMR difference spectra of MCPyV VLPs with 3'sialyllactose (3'SL) and
798 reference spectra. From top to bottom: i) VLP ^1H 1D reference spectrum; ii) STD-
799 NMR spectrum of WT VLPs with 3'SL exhibiting saturation transfer from the capsid to
800 3'SL; iii) same spectrum for Y81V mutant VLPs; iv) same spectrum with W76A
801 mutant VLPs showing no transfer to 3'SL; v) 3'SL ^1H 1D spectrum for comparison.
802 Small molecule impurities are observed in the VLP preparation (i and iv), some of
803 which are buffer components (sharp resonances with asterisks), others are
804 unidentified molecules that are likely associated with the capsids (broad resonances
805 with asterisks, the peak broadening suggests slow molecular tumbling, i.e.
806 association with the VLPs). HDO signals were truncated for the sake of visibility. B)
807 STD-NMR difference spectra of MCPyV VLPs with a GAG pentasaccharide (Arixtra,
808 Ax) and reference spectra. From top to bottom: i) VLP ^1H 1D reference spectrum; ii)
809 STD-NMR spectrum of WT VLPs with pentasaccharide exhibiting saturation transfer
810 from the capsid to the GAG; iii) same spectrum with W76A mutant VLPs; iv) GAG ^1H
811 1D spectrum for comparison. Saturation transfer to the GAG is observed for the WT

812 and the W76A mutant that does not bind to 3'SL (Fig. 8 A iv). HDO signals were
813 truncated for the sake of visibility.

814

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Figure 1

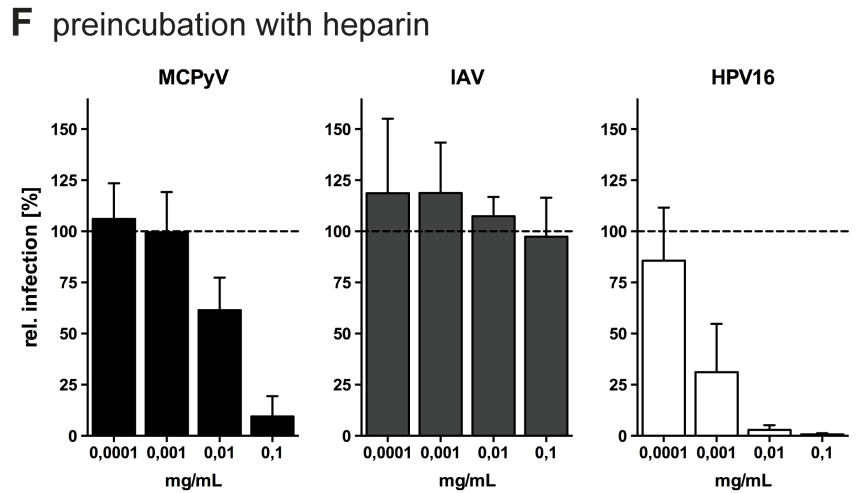
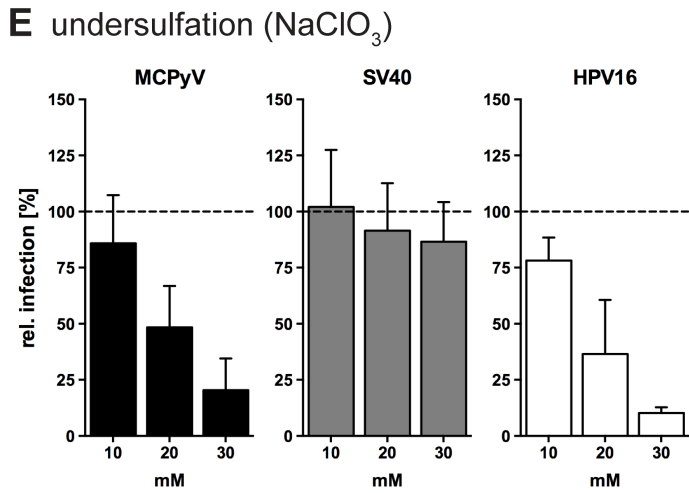
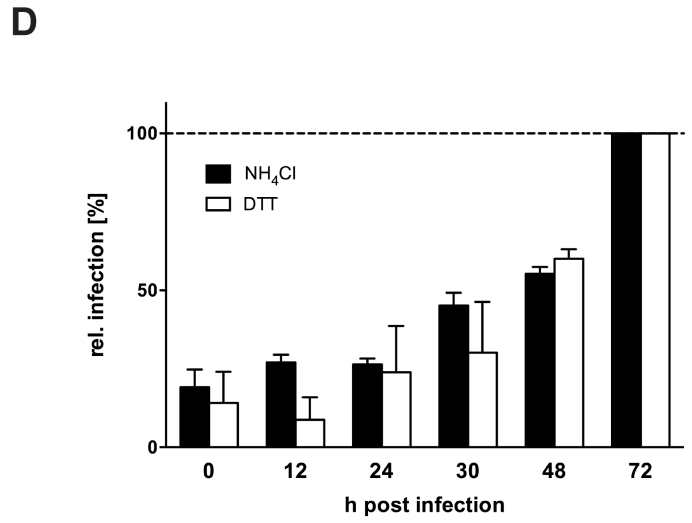
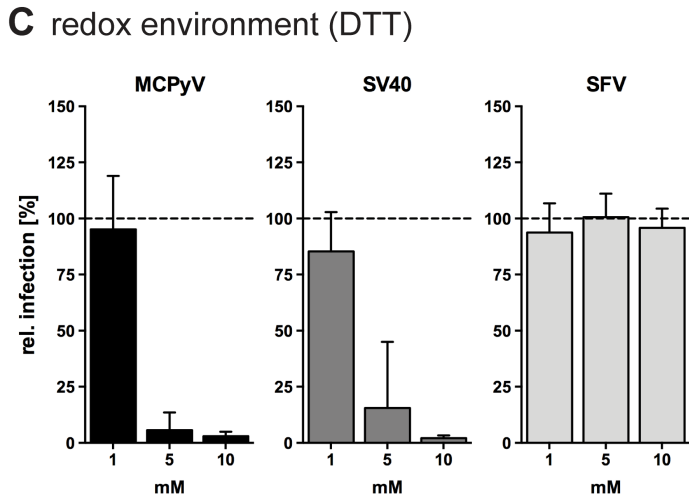
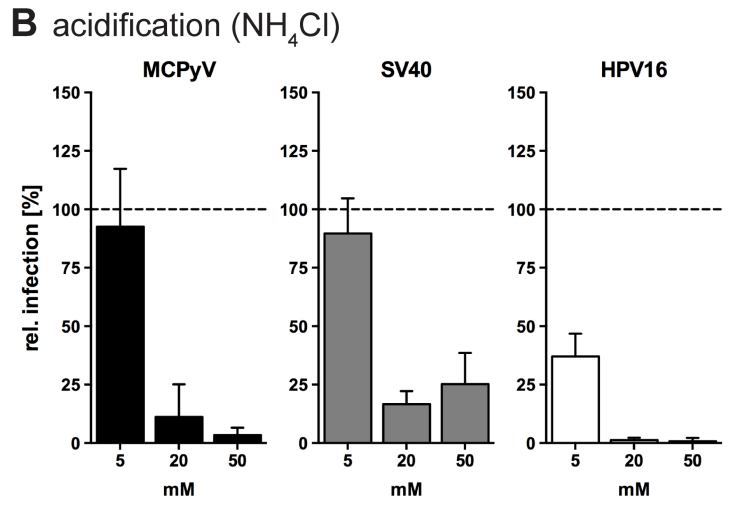
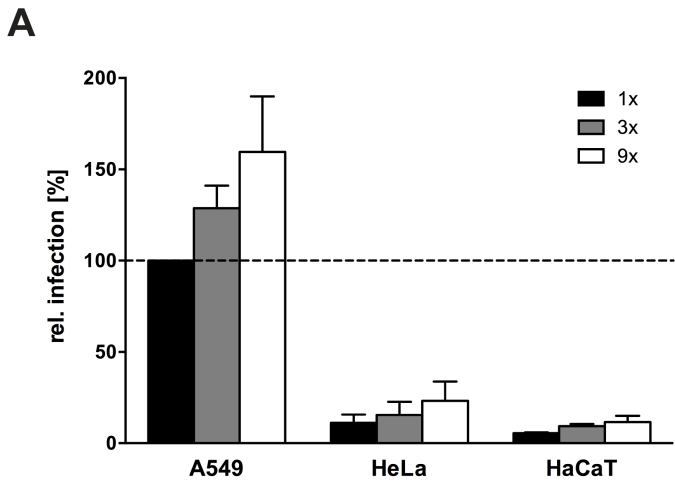
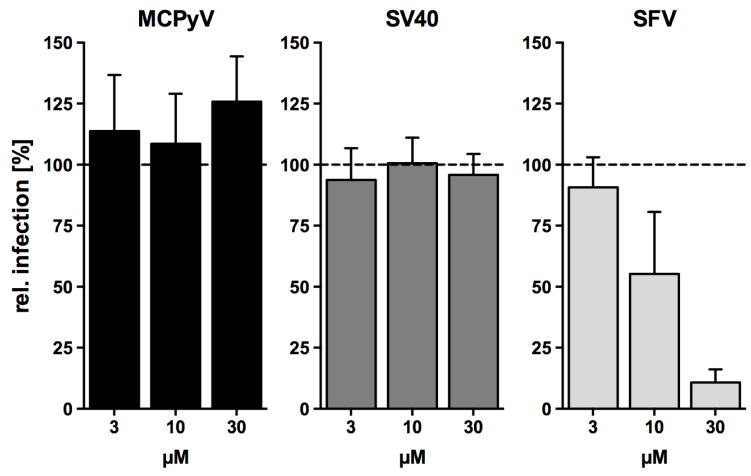
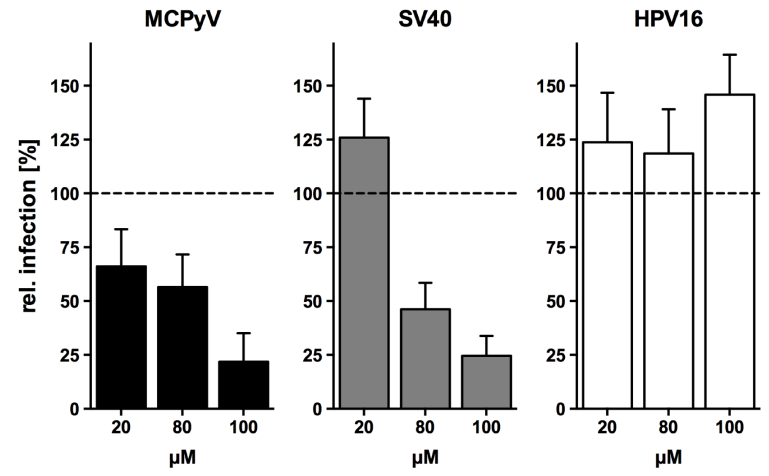


Figure 2

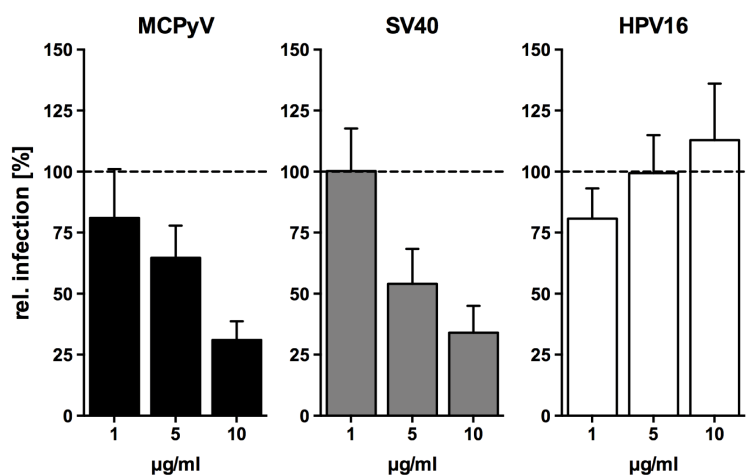
A clathrin (pitstop2)



B dynamin-2 (dynasore)



C cholesterol (nystatin/progesterone)



D Na⁺/H⁺-exchanger (EIPA)

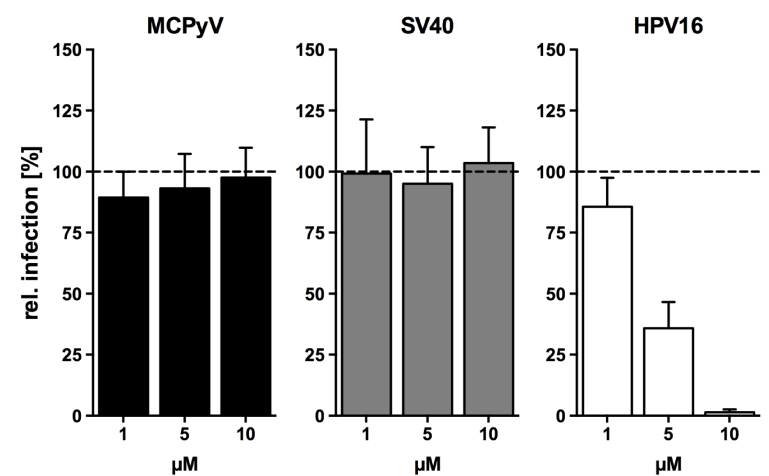
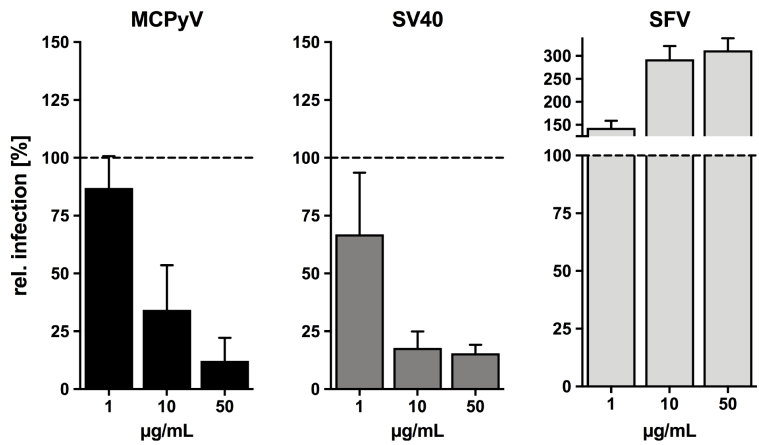
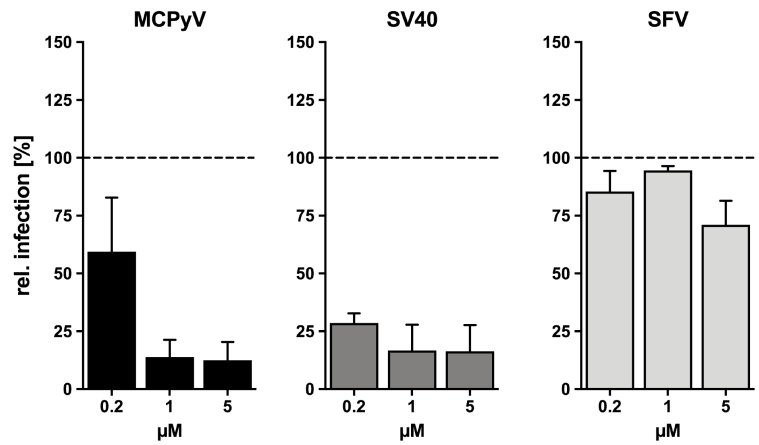


Figure 3

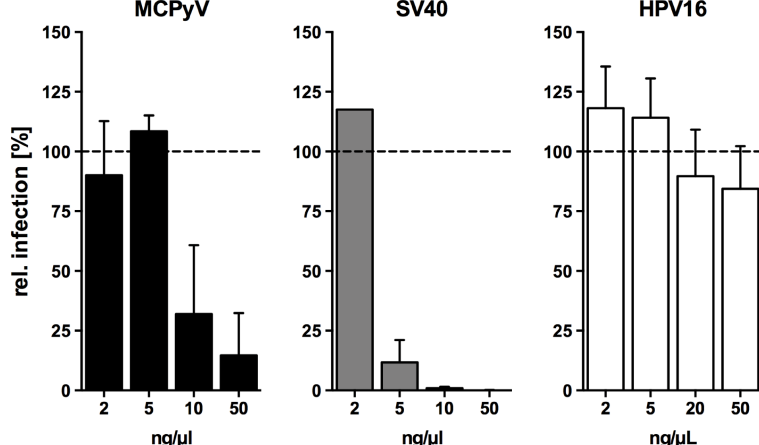
A actin (cytochalasin D)



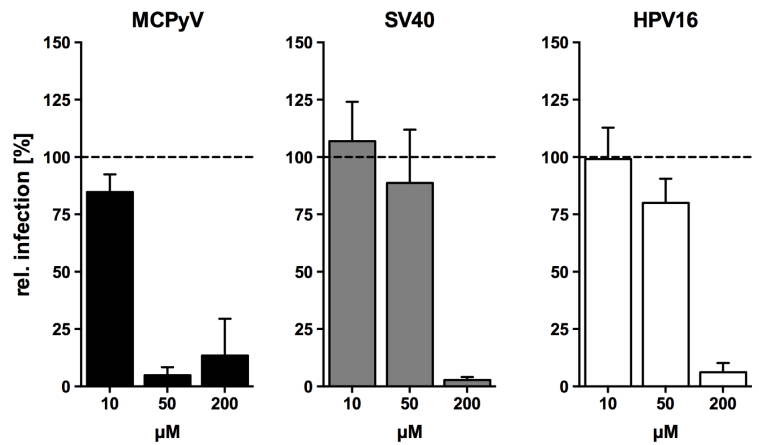
B actin (jasplakinolide)



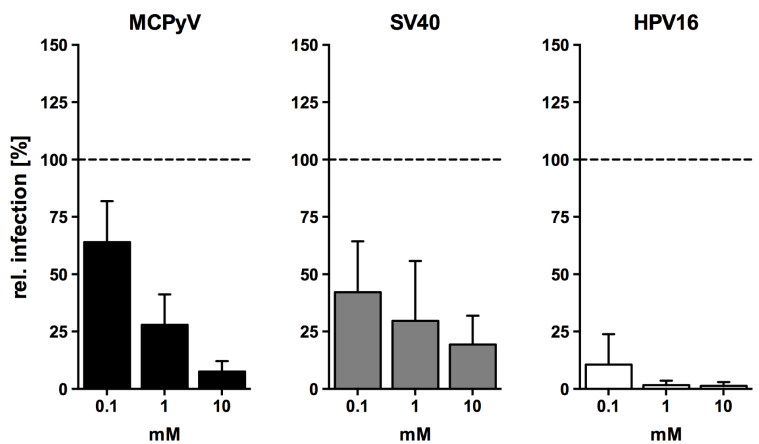
C Rho-like GTPases (toxin B)



D Tyr-kinases (genistein)



E phosphatases (sodium orthovanadate)



F PP1 and PP2A/B (okadaic acid)

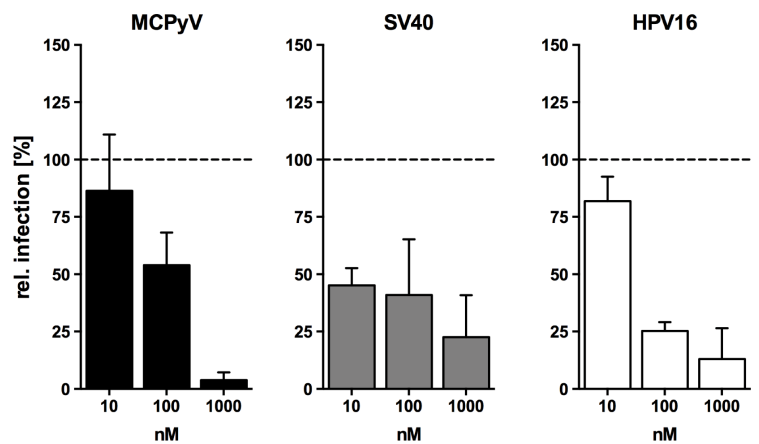
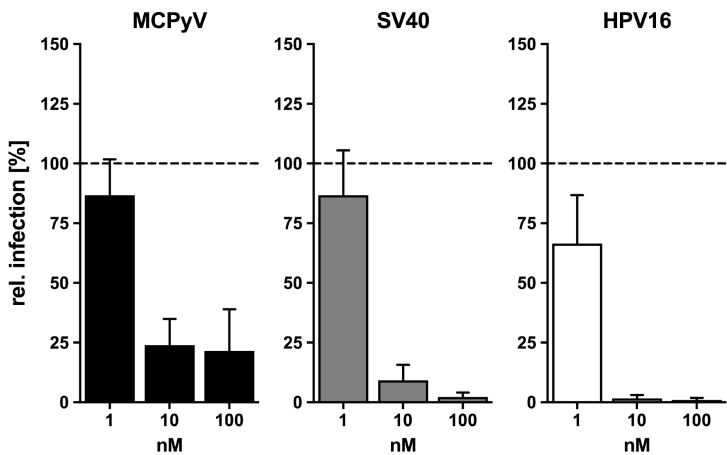
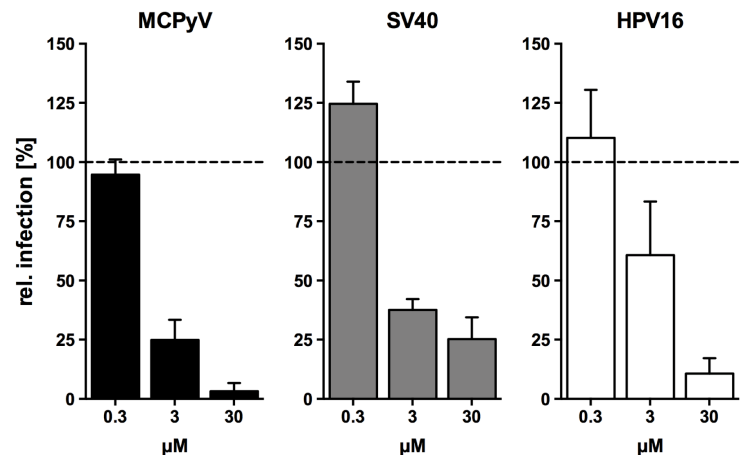


Figure 4

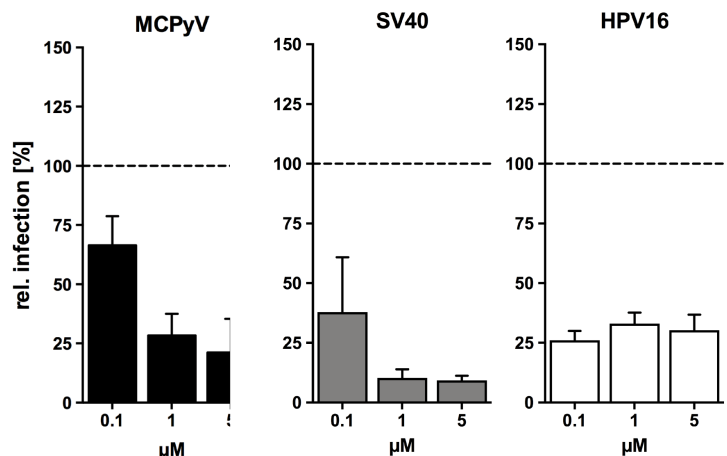
A endosomal acidification (bafilomycin A)



B microtubular transport (nocodazol)



C Golgi collapse (brefeldin A)



D cell cycle progression (aphidicolin)

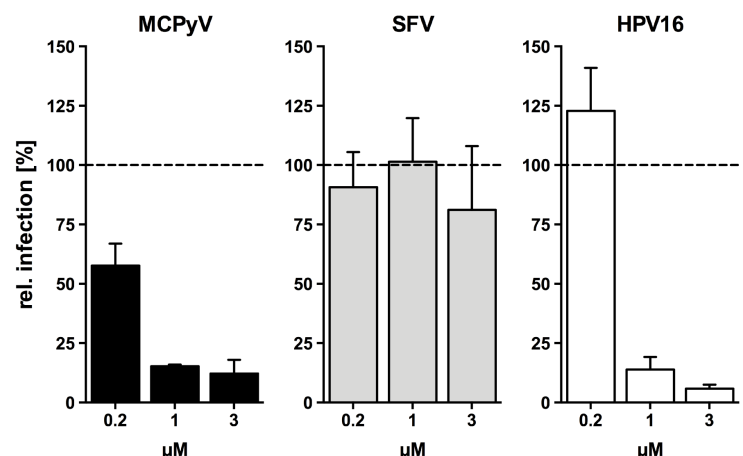


Figure 5

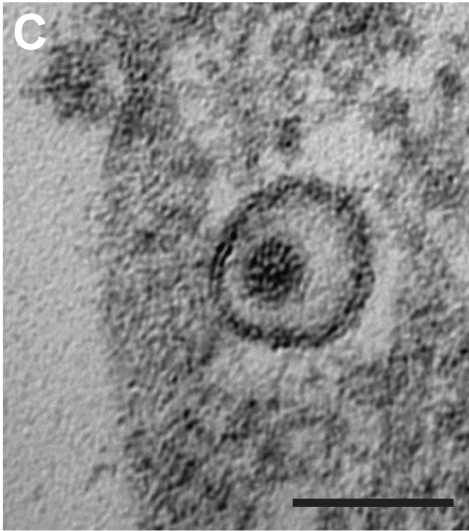
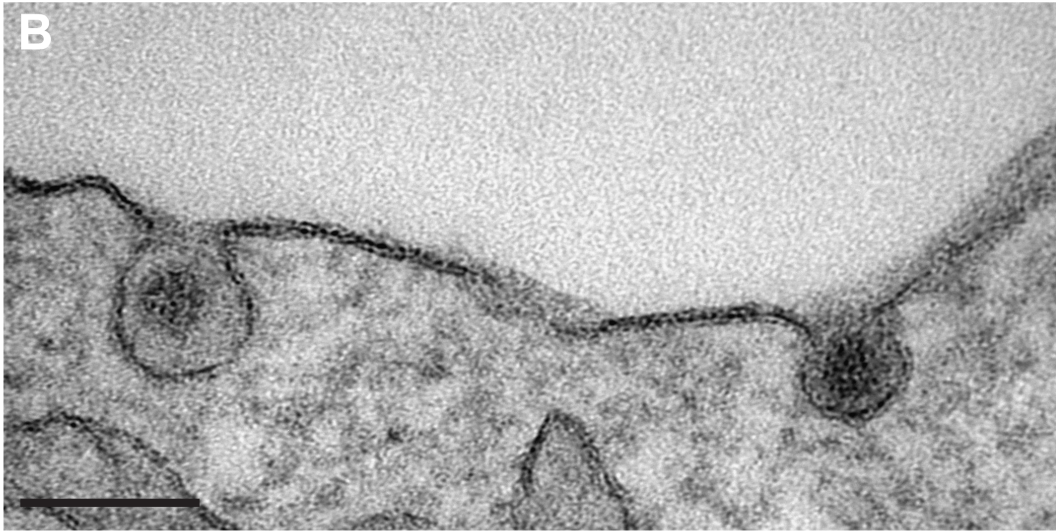


Figure 6

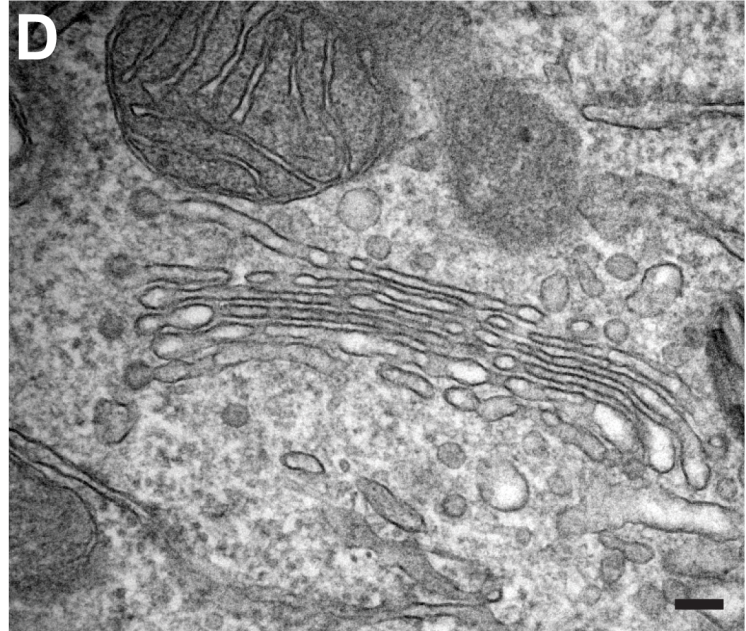
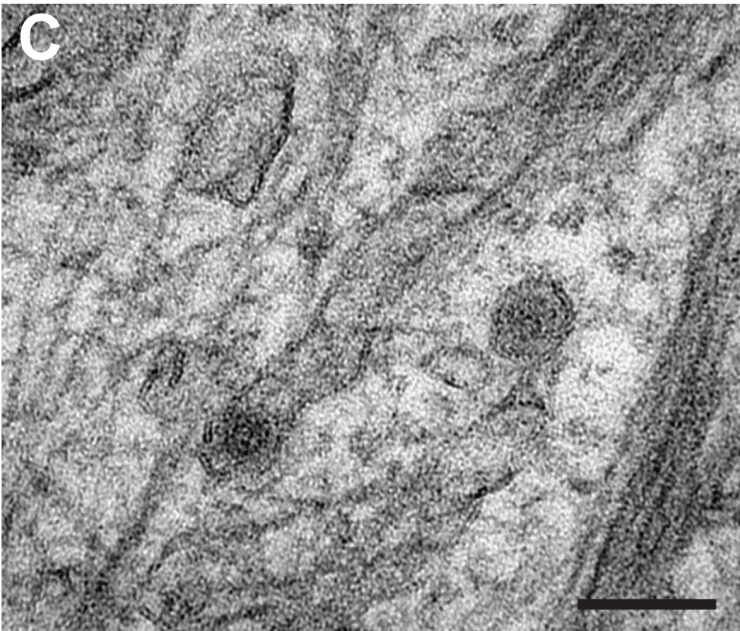
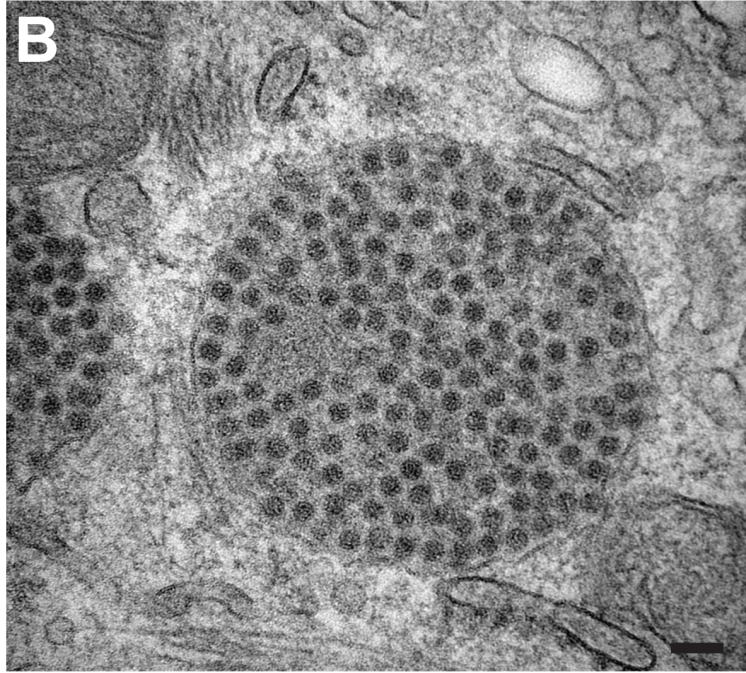
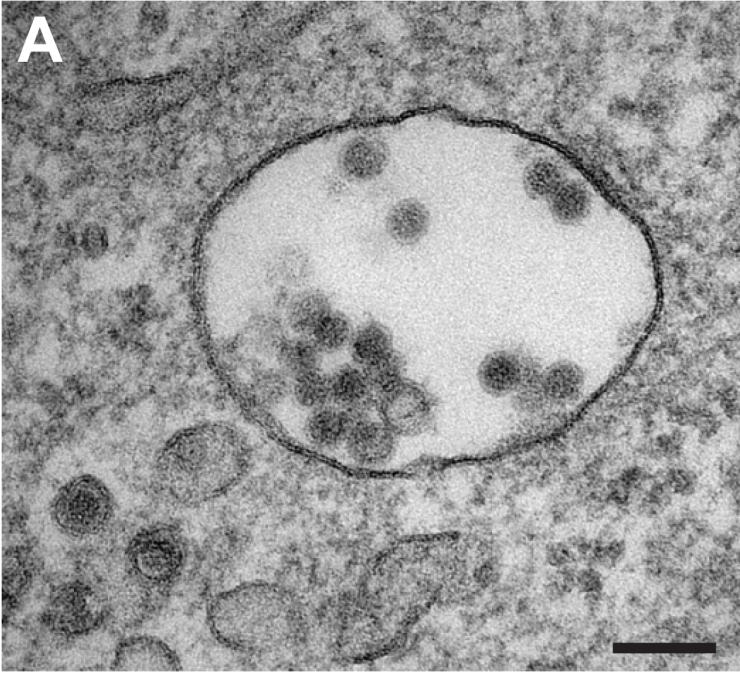


Figure 7

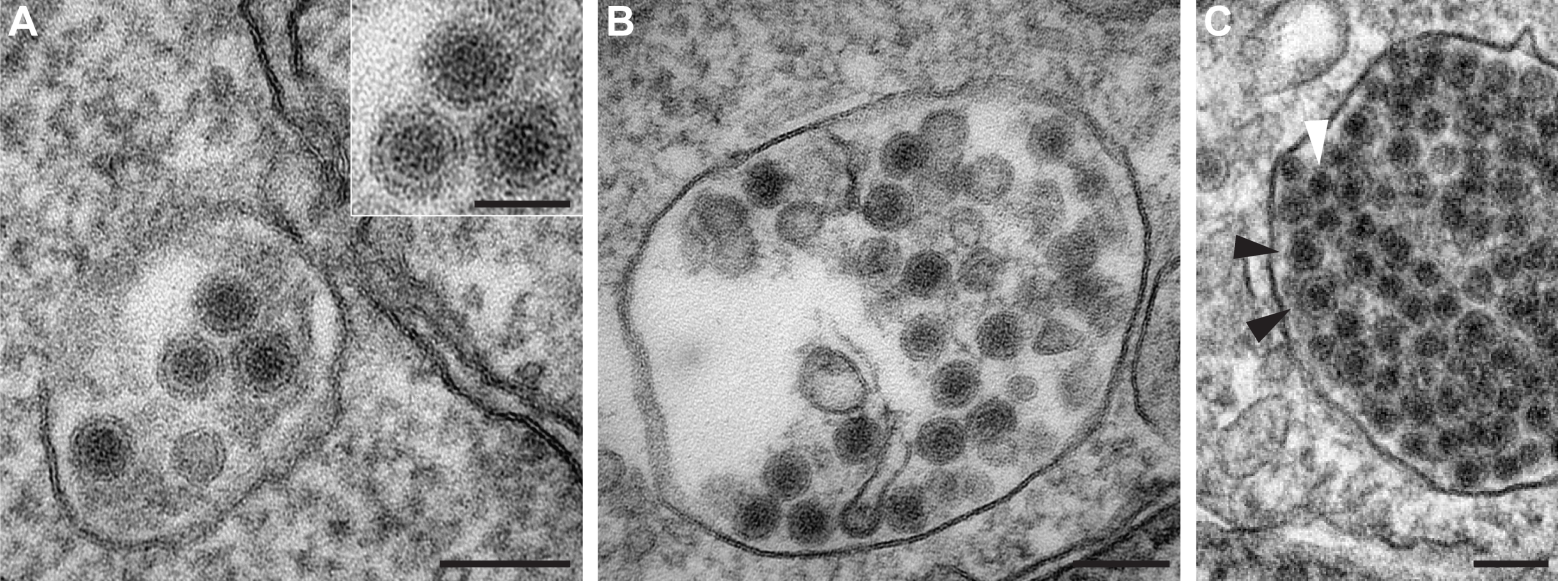
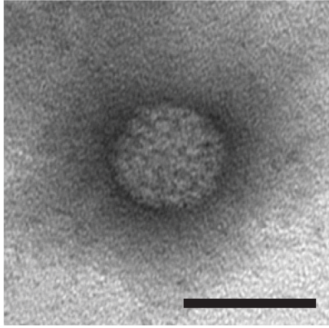
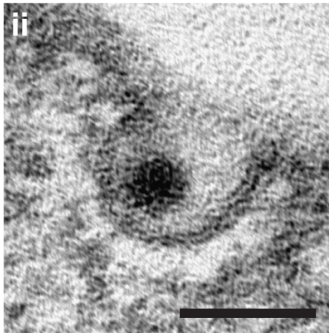
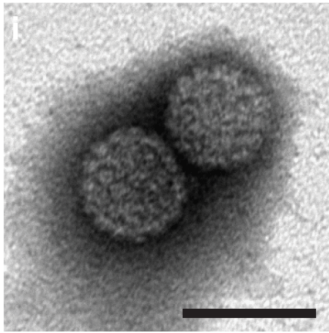


Figure 8

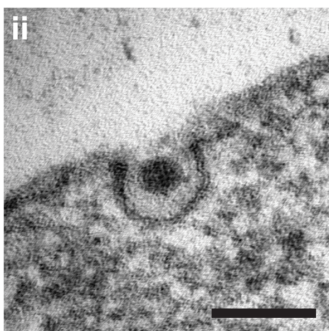
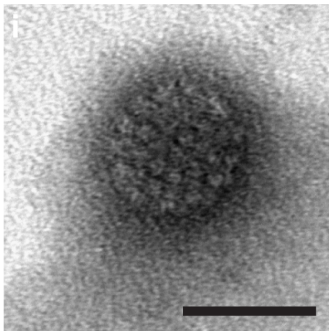
A WT



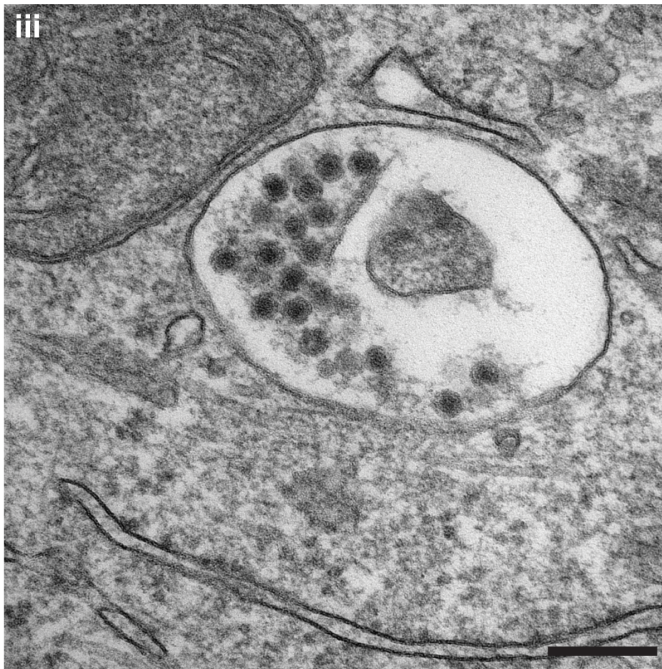
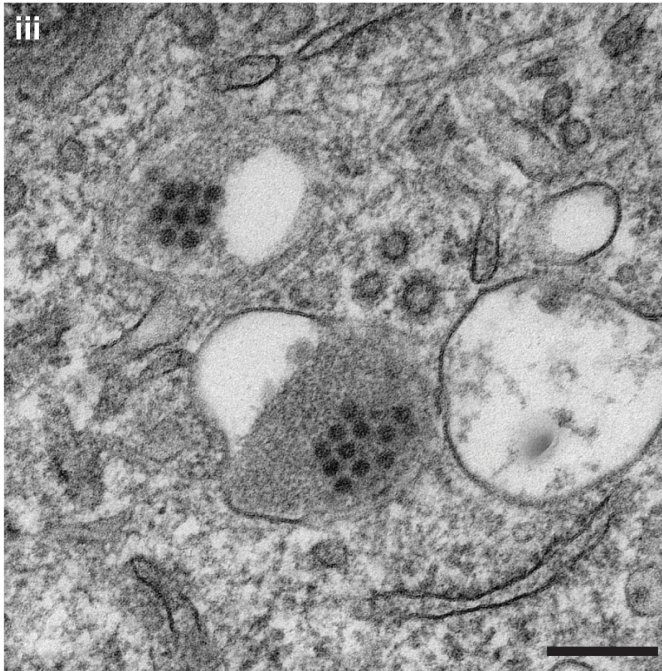
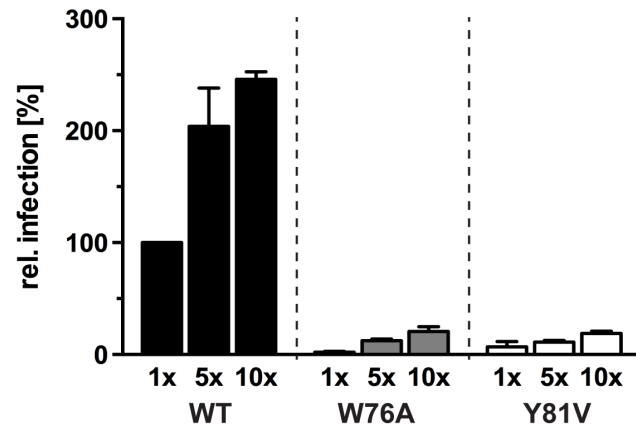
B W76A

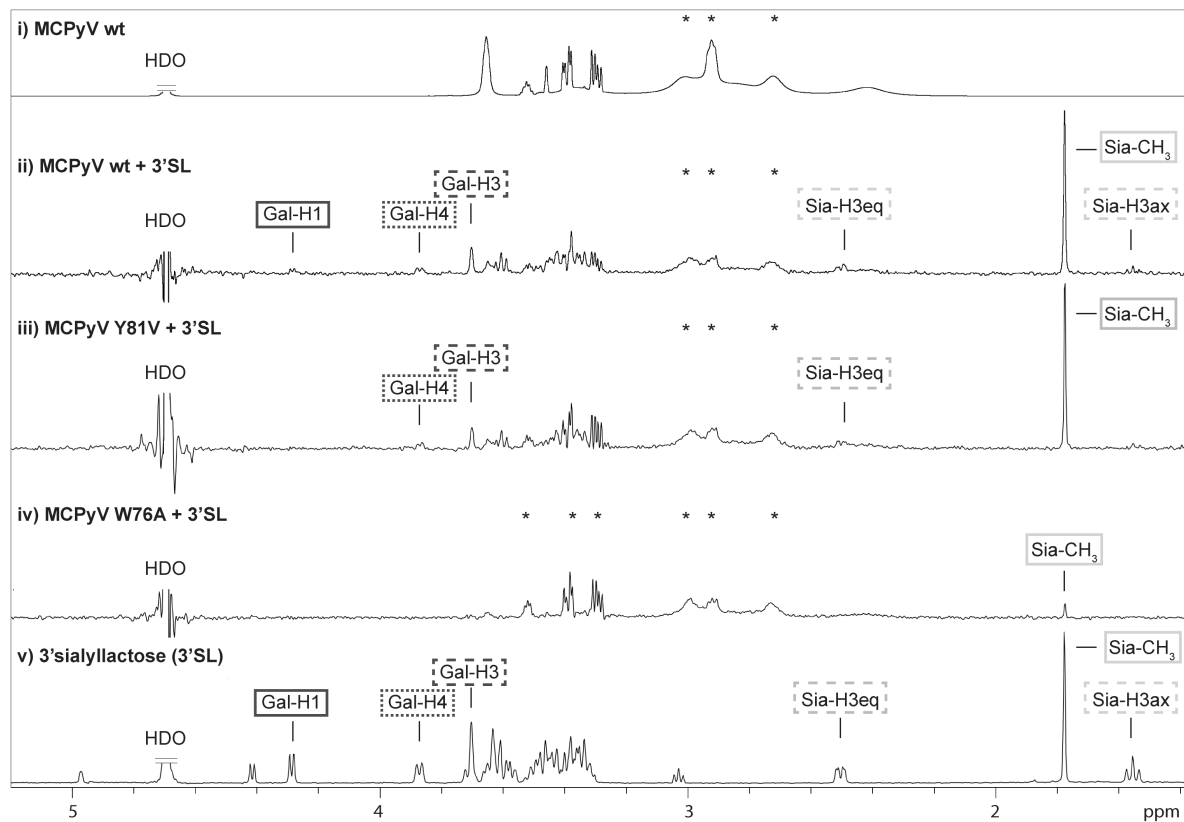
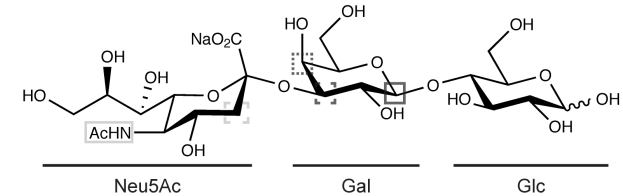


C Y81V



D



A 3'-Sialyllactose (Neu5Ac(α 2-3)Gal(β 1-4)Glc)

B Arixtra

