

1 **Essential functions of Inositol hexakisphosphate (IP6) in Murine Leukemia Virus replication**

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12 **SUMMARY**

13 We have investigated the function of inositol hexakisphosphate (IP6) and inositol pentakisphosphate
14 (IP5) in the replication of murine leukemia virus (MLV). While IP6 is known to be critical for the life cycle
15 of HIV-1, its significance in MLV remains unexplored. We find that IP6 is indeed important for MLV
16 replication. It significantly enhances endogenous reverse transcription (ERT) in MLV. Additionally, a
17 pelleting-based assay reveals that IP6 can stabilize MLV cores, thereby facilitating ERT. We find that IP5
18 and IP6 are packaged in MLV particles. However, unlike HIV-1, MLV depends upon the presence of IP6
19 and IP5 in target cells for successful infection. This IP6/5 requirement for infection is reflected in
20 impaired reverse transcription observed in IP6/5-deficient cell lines. In summary, our findings
21 demonstrate the importance of capsid stabilization by IP6/5 in the replication of diverse retroviruses; we
22 suggest possible reasons for the differences from HIV-1 that we observed in MLV.

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34 INTRODUCTION

35 The orthoretroviruses are divided into six genera (alpha-, beta-, gamma-, delta-, epsilon-, and
36 lenti-retroviruses). While the replication of viruses in different genera is similar in broad outline, there
37 are many significant differences in the details. In the present work, we describe a feature of
38 gammaretrovirus replication that differs from that of the lentivirus HIV-1, the best-studied retrovirus.

39 Retroviruses are initially assembled and released from virus-producing cells in the form of an
40 “immature” particle. Immature particles are formed from ~1500-2000 copies of the Gag polyprotein,
41 and contain the viral RNA and other proteins, all enclosed in a lipid bilayer derived from the plasma
42 membrane of the cell. After the particle has been released, it undergoes maturation, in which Gag is
43 cleaved by the viral protease into a discrete series of cleavage products. These products always include
44 the “capsid” protein (CA), which assembles within the free virion into a structure termed the “mature
45 core” or “mature capsid”. This structure encloses the viral RNA along with reverse transcriptase (RT),
46 integrase, and another Gag cleavage product, the nucleocapsid (NC) protein. In HIV-1, the mature core
47 frequently assumes a conical shape ¹.

48 In recent years, it has been recognized that a small molecule, inositol hexakisphosphate (IP6),
49 contributes to the assembly of both the immature particle and the mature core in HIV-1 ²⁻⁵. IP6 is
50 abundant in mammalian cell cytoplasm ⁶ and is packaged in immature HIV-1; its presence within the
51 virion makes it available during assembly of the mature core. In the immature particle, Gag is principally
52 arranged as a lattice of hexamers, and IP6 is coordinated within these hexamers by two rings of lysine
53 side-chains in the capsid domain of Gag (i.e., the region of Gag that will give rise to CA upon maturation)
54 in these hexamers ^{4,7-10}. Following maturation, IP6 is localized near the N-terminus of CA in the mature
55 core, now in association with basic amino acids in CA pentamers and hexamers. This association
56 profoundly increases the stability of the core, and the optimum stability appears to be essential for the
57 successful reverse transcription of the viral RNA into DNA as required for viral replication ^{4,5,11}.

58 In the present work, we have investigated the role of IP6 in the replication of the
59 gammaretrovirus Moloney MLV. We now report that IP6 is packaged in MLV, and in many respects, it
60 contributes to MLV replication in close analogy to its roles in HIV-1. However, one notable difference is
61 that the efficiency of MLV infection is reduced in target cells that are deficient in IP6 or related
62 molecules; in contrast, infection by HIV-1 is unimpeded in these cells. We propose that this difference
63 arises from a divergence between gammaretrovirus and lentivirus reproduction: gammaretrovirus cores
64 must partially disassemble in the cytoplasm of the infected cell ¹²⁻¹⁵, while lentivirus cores evidently
65 remain intact until penetrating into the nucleus ¹⁶⁻¹⁸. It seems likely that the encapsidated IP6 is lost
66 upon the cytoplasmic dissociation of MLV cores, while it is retained in HIV-1 cores.

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73 RESULTS

74 *IP6 enhances endogenous reverse transcription in MLV*

75 Reverse transcription during HIV-1 infection has been analyzed in great detail. Recent studies
76 suggest that the viral DNA is synthesized within the mature core, which remains largely or entirely intact
77 until it reaches the nucleus of the newly infected cell¹⁶⁻¹⁸. Successful infection appears to require
78 optimal stability of the core; the structure is maintained by interactions between its constituent capsid
79 (CA) molecules, as well as by small molecules associated with it^{5,11,19}. IP6 has a significant impact on
80 reverse transcription in these experiments: cores isolated from virions can synthesize viral DNA *in vitro* if
81 they are provided with optimal concentrations of IP6. In turn, the IP6 requirement is related to the
82 stability of the core, as the requisite IP6 concentration is inversely correlated with core stability in HIV-1
83 mutants^{11,20}.

84 We tested the ability of cores isolated from MLV particles to synthesize viral DNA (“endogenous
85 reverse transcription” or “ERT”). We prepared virions composed of WT MLV proteins and packaging
86 either WT MLV genomes or the genome of an MLV-derived vector encoding firefly luciferase (pBabeLuc).
87 The particles were produced in transiently transfected 293T cells and partially purified by pelleting
88 through a sucrose cushion. To measure their ERT activity, we permeabilized the virions with melittin¹¹,
89 added dNTPs and other possible cofactors, and after incubation at 37°C for 10 hours, assayed the
90 mixtures for luciferase DNA by qPCR. As shown in Figure 1A, we found that the ERT reaction was
91 promoted by the inclusion of IP6 and rNTPs, in addition to the dNTPs used in DNA synthesis. The activity
92 was also strongly stimulated by melittin and profoundly inhibited by AZTTP, as expected for a reaction
93 catalyzed by MLV RT^{21,22}.

94 As a further test of the ERT reaction, we performed PCR on the ERT products with a primer pair
95 spanning the entire luciferase coding sequence (~1.5 kbp). As seen in the lower panel of Figure 1A, the
96 synthesis of the full-length luciferase DNA was almost completely dependent upon the addition of both
97 IP6 and rNTPs, mirroring the qPCR results; it also required the melittin treatment and was sensitive to
98 inhibition by AZTTP.

99 The reaction mixtures used above contained 40µM IP6 (where indicated), a concentration
100 similar to that in mammalian cell cytoplasm⁶. It was of interest to determine the ERT activities over a
101 range of IP6 concentrations. As shown in Figure 1B, 40µM is indeed the optimal concentration under our
102 ERT assay conditions, with significantly lower yields obtained at IP6 concentrations either ≤ 10µM or >
103 160µM.

104 The ERT documented above was dependent upon the inclusion of melittin in the reaction
105 (Figure 1A). Titration showed that melittin was most effective at ~3.13-12.5 µg/ml (Figure S1A), and 6.25
106 µg/ml was used in all subsequent experiments. We also found that melittin could be replaced by Triton
107 X-100, with a threshold effective concentration of ~0.2 mM (Figure S1B). The ability of this mild non-
108 ionic detergent to replace melittin in ERT supports the hypothesis that melittin permeabilizes the viral
109 membrane, presumably giving the RT and template RNA inside the core access to the dNTPs in the ERT
110 reaction buffer, as required for DNA synthesis.

111 We also monitored the time-course of the ERT reactions in the presence and absence of IP6.
112 These experiments used primers specific for either (-) strand strong stop DNA (“early”); DNA made

113 following the first strand transfer (“intermediate”); or DNA made following the second strand transfer
114 (“late”). Note that in the assays described above, only the luciferase sequences (made by reverse
115 transcription of the pBabe-Luc luciferase vector in the virus preparations) were measured; in contrast, in
116 this kinetic experiment, the primers amplified non-coding sequences present in both the luciferase
117 vector and the intact MLV “helper” virus present in our virus preparations. As shown in Figure S1C, we
118 found that all three regions of the viral genomes were produced, as expected; the stimulation by IP6
119 was particularly significant for the later ERT products. We also noted that the amounts of “early” and
120 “intermediate” DNA decreased after the initial peak; it is conceivable that the DNase used in virus
121 purification is responsible for this decline, but we have not investigated this point.

122 Further characterization of the ERT reaction is shown in Figure S1D. Interestingly, a 5-fold
123 reduction in the concentration of the four rNTPs caused a profound decrease in ERT. Moreover, the
124 specific omission of rATP, even in the presence of all the other cofactors, drastically impaired ERT
125 product formation. Conversely, rATP alone could support ERT, even in the absence of other rNTPs.

126 In our standard ERT reaction buffer, rATP is at 6.7mM, whereas the other rNTPs are at
127 significantly lower concentrations. To test the possibility that the apparent requirement for rATP is
128 simply due to its uniquely high concentration in these experiments, we also tested ERT with the other
129 rNTPs supplied singly at 6.7 mM. As shown in Figure S1D, rCTP, like rATP, supports full ERT activity at this
130 concentration, whereas rGTP and rUTP do not. At present, we do not fully understand the rNTP
131 requirement in ERT of MLV. It seemed possible that rNTPs fulfill the same function as IP6. As shown in
132 Figure S1E, some ERT activity could be detected in the absence of rNTPs when the IP6 concentration was
133 raised approximately 10-fold beyond the optimum in the standard reaction, but even this activity was
134 far below that seen when rNTPs were included. The results suggest that rNTPs and IP6 are both
135 necessary for maximal ERT activity.

136 One possible explanation for the apparent dependence on IP6 for ERT activity (Figure 1) could
137 be that MLV RT requires IP6 for catalytic activity. We tested this hypothesis by lysing MLV particles with
138 Triton X-100 and assaying, by product-enhanced reverse transcriptase (PERT), the ability of the released
139 RT to copy an external template, i.e., MS2 RNA, into DNA by qPCR. As shown in Figure S1F, varying the
140 IP6 concentration between 0 and 200 μ M had no significant effect on the reaction. Thus, IP6 promotes
141 ERT (Fig. 1A) by an effect on the cores, not on the RT enzyme *per se*.

142 *Other cyclic polyanions also promote ERT in MLV*

143 We also tested other small molecules for their ability to replace IP6 in the ERT assay. As shown
144 in Figure 2, we found that several cyclic polyanions, i.e., inositol pentakisphosphate (IP5) (Figure 1C),
145 inositol hexasulfate (IS6) (Figure 1D), and hexacarboxybenzene (HCB or mellitic acid) (Figure 1E), could
146 all promote ERT. Interestingly, IS6 was nearly as active as IP6, while IP5 and HCB were somewhat less
147 active. In contrast, the uncharged cyclic molecule inositol was completely inactive (Figure 1F).

148 *IP6 stabilizes MLV cores*

149 In HIV-1, the stability of the core in permeabilized or lysed virus preparations has a profound
150 effect upon ERT activity, and IP6 appears to promote ERT by stabilizing the cores^{11,20}. We therefore
151 tested the ability of IP6 to stabilize MLV cores. MLV particles were incubated for one hour at 37 °C with
152 melittin in the presence or absence of co-factors tested in Figure 1. The reactions were then centrifuged

153 through a sucrose cushion and the amount of p30^{CA} in the pellet was analyzed by immunoblotting. The
154 results are shown in Figure 2A and the recovery of p30^{CA} in the pellet quantitated in Figure 2B. It is
155 evident that the three active additives, i.e., IP6, IS6, and HCB, all strongly protected the MLV cores from
156 disruption by melittin, as the majority of the CA protein remained pelletable in their presence, but not in
157 inositol (In).

158 To further characterize the viral components in the ERT reactions, we also examined the pellets
159 by transmission electron microscopy. As shown in Figure 2C, mature viral cores were visible in the
160 pellets obtained after treatment of the particles with melittin and the polyanions IP6, IS6, or HCB. In
161 contrast, the pellets produced in the absence of melittin contained intact mature virions (as expected),
162 while those isolated in melittin alone or with the inactive additive inositol contained few if any mature
163 particles or cores; immature particles were occasionally seen in these samples. Taken together with the
164 immunoblotting results in Figure 2A and 2B, the data indicate that the active additives stabilize the cores
165 within mature MLV particles, in close analogy with prior results on HIV-1^{11,20}.

166 We also tested a wide range of IP6 concentrations in the pelleting assay. Remarkably, the
167 protection against disruption of the cores appeared to be a monotonic function of the IP6
168 concentration: at 2.4 mM the recovery of p30^{CA} in the pellet was virtually complete (Figure S2A and
169 S2B). This is in striking contrast with the ERT results: as noted above (Figure 1B), IP6 concentrations of
170 40-80 μ M were optimal for ERT, and those above 160 μ M were strongly inhibitory. It thus appears that
171 efficient ERT depends upon an intermediate level of capsid stability. Again, these results are quite
172 analogous to previously published findings on HIV-1¹¹.

173 We also tested the ability of rNTPs to stabilize the cores in the pelleting assay. As shown in
174 Figures S2C and S2D, each rNTP was able to stabilize the cores. This is also notable for its contrast with
175 the ERT results, in which (Figure 2) rATP and rCTP were active, while rGTP and rUTP were not. The
176 contrast implies that the enhancement of ERT by rNTPs is not a result of stabilization of the cores.

177 *MLV packages IP5 and IP6*

178 In light of these striking effects of exogenous IP6 and related molecules on ERT activity, it was of
179 interest to determine whether MLV, like HIV-1^{3,4}, packages IP6. We prepared MLV particles and assayed
180 them for IP5 and IP6 as described in Star Methods. Control preparations, prepared in parallel with the
181 MLV, included HIV-1 and “mock” virus preparations, which were generated from supernatants of
182 cultures transfected with an empty expression vector. As shown in Figure 3A, we found that IP6 was
183 present in the MLV preparations. As the IP6 levels were significantly higher than that in the “mock”
184 sample, the data strongly indicate that IP6 is an authentic component of MLV particles, rather than a
185 background in the assay. IP5 was also detected in the MLV preparations (Figure 3C). The ratios of IP6
186 and IP5 to CA protein tended to be somewhat lower in MLV than in HIV-1 (Figure 3B and 3D).

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188 *Possible role of R3 residue of MLV capsid in interacting with IP6*

189 In immature HIV-1 particles, IP6 is localized in the centers of Gag hexamers, with a ring of lysines
190 (CA residue 158) above and another ring of lysines (CA residue 227) below the IP6, while in mature
191 particles IP6 is coordinated by a ring of arginines (CA residue 18) in a CA hexamer^{3,4}. It was of interest to
192 try to determine the location of the IP6 within MLV particles. A careful examination of mature MLV

193 using cryo-EM tomography²³ previously noted “a faint additional density” within the hexameric N-
194 terminal domain of the CA region of mature particles, surrounded by six arginine side-chains (one from
195 each monomer in the hexamer) of CA residue R3.

196 It seemed possible that this unidentified density could be IP6. We note that the R3 residue
197 appears to be absolutely conserved in gammaretroviruses (Figure S3A); this conservation suggests that
198 R3 is essential for optimal viral replication and is consistent with a role in packaging of the important
199 cofactors IP5/6. To further test the significance of R3 in MLV, we analyzed the properties of MLV
200 mutants in which it was replaced with lysine or alanine. We found (Figure 4A and 4B) that mature R3K
201 and R3A virions contain IP5 and IP6 levels indistinguishable from those of WT virions; thus, R3 is not
202 necessary for packaging of IP5/6 into virions. On the other hand, we found (Figure 4C) that these
203 mutants lack any detectable infectivity although cells transfected with R3 mutant MLV constructs
204 produced comparable levels of viruses to WT MLV (Figure 4D). In addition, we did not observe any
205 defect in proteolytic processing of Pr65 Gag in the cell lysates of cells transfected with either of the
206 mutants (Figure 4D). We also examined the mutant particles by TEM. We found, just as in WT MLV, two
207 types of virus morphology: one resembling immature MLV, with a ring of density under the virus
208 membrane, and the other with density in the center of the particle, as in mature MLV (Figure S3B). Thus,
209 the mutations did not cause any discernible change in the overall morphology of the particles.

210 As the mutants were comparable to the WT in their ability to release viruses, we wanted to test
211 if the mutant viral cores are also dependent on IP6 for DNA synthesis *in vitro* using our ERT assay.
212 Interestingly we observed that the R3 mutant virions are apparently incapable of ERT, even in the
213 presence of IP6 (Figure 5A). We also tested the ability of IP6 to stabilize cores from R3 mutant virions;
214 as shown in Figure 5B and 5C, they also differ from WT cores (see Figure 2) in that they are not stabilized
215 by IP6. These results imply that R3 is required for core stabilization by IP6 and are consistent with the
216 hypothesis that this interaction with IP6 is essential for ERT.

217 *IP6 functions in MLV replication*

218 While the data presented above demonstrate the effects of IP6 in *in vitro* assays of MLV
219 function, it was obviously of interest to determine the role, if any, of IP6 in viral replication in
220 mammalian cells. We explored this question by testing viral replication in cells that were deficient for
221 IP6 and for IP5. These experiments utilized 293T cells in which the inositol polyphosphate multikinase
222 (IPMK), which synthesizes IP5, or the inositol pentakisphosphate 2-kinase (IPPK), which synthesizes IP6,
223 had been knocked out by CRISPR/Cas9 technology²⁴. It has been shown by two independent studies
224 that upon knockout (KO) of IPMK, levels of both IP5 and IP6 are significantly reduced. However, for the
225 IPPK-KO, IP6 levels were significantly reduced, but IP5 levels were seen to be elevated^{24,25}. We also
226 confirmed these results using direct biochemical assays for IP5 and IP6 and observed that both IPMK-
227 and IPPK-KO cells had lower levels of IP6 than the WT control cells. IPMK-KO cells had a pronounced
228 reduction in IP5 levels, while the IPPK-KO cells only had a modest reduction (Figure S4)

229 Cultures of the KO and control cells were transfected with plasmids encoding MLV Gag and Gag-
230 Pol; xenotropic MLV Env; and the MLV-based luciferase vector (pBabeLuc). 48 hrs after transfection, the
231 cells were lysed and supernatants were collected for virus assays. Western blot analysis of the cell
232 lysates and supernatants (Figure 6A and Figure S5A, 5B and 5C) showed that expression of viral genes
233 and the rate of virus production were substantially reduced in the KO cells; however, the expressed Gag
234 proteins were assembled into released virions with an efficiency similar to that in the WT control cells

235 (Figure 6B). Significantly, the specific infectivity of the virus released from the KO cells was virtually the
236 same as (or, in the case of the IPMK-KO cells, slightly higher than) that from the control cells (Figure 6C).
237 Thus, normal levels of IP5 and IP6 in virus-producing cells are not necessary for the production of fully
238 infectious MLV.

239 We also tested the possibility that the reduction in IP5 and IP6 levels might render the KO cells
240 less infectable than WT control cells. Parallel cultures of the KO and control cells were infected with
241 virus preparations containing both MLV (with a xenotropic MLV Env) and the MLV-based luciferase
242 vector, and infection was assessed by measuring luciferase activity in the infected cultures. The
243 luciferase activity in the cell lysates was normalized to β -actin levels in the lysates to correct for
244 differences in cell density. We found a highly reproducible reduction in infections in the KO cells (Figure
245 6D), with a larger decrease in IPMK-KO than in IPPK-KO cells.

246 It has been reported previously^{5,25} that IP5/6 deficiency in the target cells does not significantly
247 affect their infectability by HIV-1. Our results with MLV (Figure 6D) are in direct contrast with these
248 findings with HIV-1. To confirm this difference between the viruses, we also checked the infectability of
249 the KO cells with an HIV-1-based luciferase vector. The same MLV Env was used in this pseudotype as in
250 the MLV experiments shown in Figure 6D. As shown in Figure 6E, the KO of IPMK and of IPPK did not
251 reduce the efficiency with which the cells could be infected with HIV-1, in agreement with prior studies
252^{5,25}.

253 The data presented above indicate that KO cells produce normal, fully infectious MLV, while the
254 efficiency with which they can be infected by MLV is somewhat reduced. As an additional test of this
255 conclusion, we tested the ability of MLV produced from KO cells to infect the KO cells. As shown in
256 Figure S5D and S5E, this virus exhibits the same profile as the virus produced in WT cells (see Fig. 6D): a
257 modest but highly reproducible reduction in infections in the KO cells, particularly the IPMK-KO cells.
258 This confirms that the reduced IP5/6 levels in the virus-producing cells have no significant effect upon
259 the dependence of the virus upon IP5/6 levels in the target cells.

260 To further confirm that the reduced infection of the KO cells resulted from the lack of the IPMK
261 and IPPK, we tested whether transient transfection with expression vectors for the missing enzymes
262 could reverse the effect. As shown in Figure 6F, the defect in infectability of the IPMK-KO cells was
263 almost completely reversed by either of the kinases, while there was little effect in the IPPK-KO cells,
264 whose infectability is much closer to that of the control cells than that of the IPMK-KO cells.

265 We also tested the ability of another enzyme, multiple inositol polyphosphate phosphatase
266 (MINPP1), to reduce the infectability of target cells. This enzyme converts IP6 to IP5 and IP5 to IP4,
267 reducing cellular levels of both IP6 and IP5^{26,27}. Using the same methodology as in Figure 6F, we
268 transfected the control and KO cells with an expression plasmid for MINPP1 and challenged them with
269 the MLV preparations containing the luciferase vector. As shown in Figure 6G, we found that this
270 treatment reduced infection of the IPPK-KO cells, while we could not detect an effect in the control cells
271 or the IPMK-KO cells. As part of this experiment, we also checked the expression of MINPP1 in the
272 transfected cells by immunoblotting. Interestingly, we found (Figure S6) that the enzyme was at a
273 substantially higher level in the IPMK-KO cells than in the IPPK-KO cells or control cells. This unexpected
274 observation raises the possibility that MINPP1 expression in 293T cells is controlled by IP5 or IP6 levels,
275 but we have not explored this further. In any case, the fact that MINPP1 reduced the infectability of

276 IPPK-KO cells further supports the conclusion that infection by MLV depends upon the maintenance of
277 proper IP5 and/or IP6 levels in target cells.

278 *Reduced infectability of KO cells is reflected in reduced viral DNA synthesis*

279 We also tested whether the rate or amount of viral DNA synthesis is reduced upon infection of
280 the KO cells. The KO and control cells were infected with MLV preparations (containing both intact MLV
281 and the luciferase vector) and were lysed at 3, 6, and 10 hours after infection. The lysates were assayed
282 for early, intermediate, and late DNA products, as well as luciferase DNA, by qPCR. They were also
283 assayed for the cellular gene CCR5, which was used to normalize the lysates for differences in cell
284 numbers. As shown in Figure 7, in all cases reverse transcription was impaired in the KO cells. The defect
285 was most severe in late DNA synthesis and was greater in the IPMK-KO cells than in the IPPK-KO cells,
286 mirroring the relative infectability of the cells (Figure 6D).

287 **DISCUSSION**

288 In the last few years, the small molecule IP6 has been found to play a significant role in HIV-1
289 replication⁷. It is present at substantial levels within virions. It both promotes assembly of the immature
290 particle, and enhances the stability of the core within mature virions. This stabilization appears to
291 facilitate DNA synthesis within the core in newly infected cells¹¹.

292 In the present work, we have investigated the participation of IP6 in the replication of MLV, a
293 member of the gammaretrovirus genus. Our results can be briefly summarized as follows: just as in HIV-
294 1, IP6 is packaged in MLV particles. In assays of ERT (the copying *in vitro* of viral RNA to DNA by the RT in
295 viral lysates), we found that adding IP6 profoundly enhances DNA synthesis. Also in analogy with prior
296 work on HIV-1^{11,20}, this enhancement reflects the stabilization of the structure of the viral core by
297 exogenously supplied IP6 in particles permeabilized by the pore-forming peptide melittin or,
298 alternatively, lysed by the mild nonionic detergent Triton X-100. Finally, cell-based infection assays
299 reveal that MLV infection depends on the presence of IP6 and IP5 in target cells, while in the case of
300 HIV-1, such dependence is absent.

301 Our analysis of the contribution of IP6 to MLV replication was made possible by the existence of
302 cell lines from which the two biosynthetic enzymes, IPPK and IPMK, had been (separately) knocked out
303²⁴. Briefly, we found that when IPPK-KO and IPMK-KO cells were transfected with plasmids encoding
304 infectious MLV, they produced virus with unimpaired specific infectivity, albeit at a lower level than the
305 control cultures. This is analogous to previously published findings on HIV-1^{24,25}.

306 On the other hand, infection of IPMK-KO or IPPK-KO target cells with normal MLV was
307 somewhat less efficient than infection of controls; this is clearly contrary to results with HIV-1^{5,25}, which
308 we confirmed as part of our study. It is interesting to note that the defect in infectability was greater in
309 the IPMK-KO cells than the IPPK-KO cells, that the low infectability of the IPMK-KO cells could be
310 reversed by transient transfection of plasmids encoding either IPMK or IPPK into the cells, and also that
311 the infectability of the IPPK-KO cells was further reduced by transient transfection of a plasmid encoding
312 MINPP1, a phosphatase that reduces the phosphorylation of IP6 and related compounds^{26,27}. (The
313 experiments with MINPP1 also strongly suggested that steady-state levels of this enzyme in 293T cells
314 may be negatively regulated in response to IP5, but we have not explored this observation further.) This

315 target-cell effect is the one fundamental difference we have found between MLV and HIV-1 with respect
316 to IP6 function. We consider its significance below.

317 Several observations in this study should also be noted. In addition to IP5 and IP6, the small
318 cyclic polyanions IS6 and HCB also promote ERT in MLV. While HCB and IP5 are somewhat less active on
319 a molar basis than IP6, IS6 is nearly equivalent to IP6 in this assay. This is rather unexpected as IS6
320 carries significantly less negative charge than IP6. In any case, these charges are apparently essential for
321 promotion of ERT, as inositol, the uncharged parent of IP6 and IS6, appears to be completely devoid of
322 activity (Figure 1).

323 We also found that rNTPs are necessary for the ERT reaction. One might imagine that they serve
324 the same function in the reaction as IP6, as they are also small polyanions, but this does not seem to be
325 the case: our experiments gave no indication that there was redundancy in these requirements. In fact,
326 further analysis showed that the “rNTP” requirement can be fulfilled by rATP or rCTP, but not, as far as
327 we could determine, by rGTP or rUTP (Fig. S1). We do not know how these specific nucleoside
328 triphosphates contribute to ERT, but it is particularly surprising that there is base-specificity in the
329 reaction and that of the two active rNTPs, one has a purine and the other a pyrimidine base. The
330 contribution of rNTPs to ERT also cannot be explained by an ability to stabilize the core, as all four rNTPs
331 possessed this activity (Fig. S2), but, as noted here, only rATP and rCTP supported ERT. In contrast, in
332 HIV-1 it has been found that either rATP or rGTP can protect disulfide-stabilized CA hexamers against
333 thermal denaturation ⁴; rATP also enhances ERT in HIV-1 ⁴.

334 Our experiments also showed that IP6 imparts some degree of stability to cores isolated from
335 MLV virions and that this stability seems necessary for the synthesis of viral DNA *in vitro*. On the other
336 hand, supraoptimal levels of IP6 apparently produce hyperstable cores which cannot synthesize DNA
337 efficiently, in further resemblance to HIV-1 ¹¹. It seems likely that just as with HIV-1 ^{11,20}, addition of IP6
338 will be very helpful in the purification of MLV cores from mature virions.

339 We also addressed the question of the location of IP6 within mature MLV cores. Qu et al. ²³ had
340 reported that a ring of six arginines at residue 3 of MLV CA appears to coordinate an unidentified
341 density; it seemed possible that this density is IP6. We tested the significance of this residue by replacing
342 it with lysine or with alanine. These mutants possess no detectable infectivity (Fig. 4) and are evidently
343 incapable of ERT, even in the presence of IP6 (Fig. 5). IP6 also fails to stabilize their cores in melittin-
344 disrupted particles (Fig. 5). All of these findings are consistent with the hypothesis that IP6 is
345 coordinated by R3 in mature MLV cores, although other explanations cannot be excluded. We also
346 found that these mutant virions still contain IP6 (Fig. 4); thus, R3 is not necessary for IP6 packaging.

347 Why should wild-type levels of IP5 and/or IP6 in the target cells be required for successful
348 infection by MLV, when this is not the case for HIV-1? One key difference in the replication of the two
349 viruses is that unlike HIV-1, MLV cannot infect non-dividing cells ^{13,28}. Recent studies have shown that in
350 HIV-1, the core of the mature infecting particle remains partly or entirely intact while it is in the
351 cytoplasm and copying its RNA into DNA ^{16-18,29,30}. The core is capable of penetrating the nucleus by
352 interacting with cellular nuclear-import machinery, and only dissociates and releases the viral DNA
353 (complexed with integrase [IN]) within the nucleus. In MLV, in contrast, the DNA cannot penetrate the
354 interphase nucleus. Rather, the core at least partially disassembles within the cytoplasm, and a complex
355 of DNA and IN, together with the Gag fragment p12 as well as CA, gains access to nuclear DNA by
356 binding to mitotic chromosomes in dividing cells ¹²⁻¹⁵.

357 We propose that because MLV cores disassemble in the cytoplasm during infection, they are
358 dependent upon the cellular IP6 for maintaining the structural stability required for efficient reverse
359 transcription. Cores of HIV-1 particles, however, bring IP6 with them and never relinquish it during their
360 journey to the nucleus. Perhaps HIV-1 cores have a higher affinity for IP6 than MLV cores. In any case,
361 this would explain why the reduction of IP6 levels in target cells does not impair HIV-1 infection. The
362 nature of the “trigger” leading to the disassembly of MLV cores, but not HIV-1 cores, in the cytoplasm of
363 newly infected cells remains to be determined.

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371 **AUTHOR CONTRIBUTIONS**

372 Conceptualization, B.B., K.K.L., S.K.D and A.R.; Methodology, B.B., K.K.L., H.B., S.K.D., D.H., G.A.S., C.A.,
373 and A.R.; Investigation, B.B., K.K.L., H.B., S.K.D., D.H., C.A., and A.R; Writing – Original Draft, B.B and A.R.;
374 Writing – Review & Editing, A.R.; Funding Acquisition, A.R. and C.A.; Resources, A.R. and C.A.;
375 Supervision, S.K.D., C.A., and A.R.

376 **DECLARATION of INTERESTS**

377 The authors declare no competing interest.

378 **FIGURE TITLES AND LEGENDS**

379 **Figure 1: IP6 promotes ERT in MLV:** A) The bar graph shows ERT product formation in the presence and
380 absence of different reagents as indicated in the x-axis of the graph. The y-axis represents absolute
381 copies of luciferase DNA reverse transcribed from the MLV-derived luciferase vector RNA packaged in
382 MLV. In one sample, 30 μ M AZTTP was included in the reaction mixture. Below the bar graph is an
383 agarose gel showing amplification of a longer product (luciferase coding sequence, 1500bp) after ERT.
384 NTC- no template control for the PCR reaction. B) IP6 titration in ERT assay. The graphs represent the
385 mean \pm SD of three replicates in the qPCR measurement of a single experiment selected from at least
386 two independent experiments with similar results. C)-F): ERT product accumulation by titrating different
387 potential co-factors in place of IP6: C) IP5, D) IS6, E) HCB (Mellitic acid), and F) Inositol.

388

389 **Figure 2: Cyclic polyanions stabilize MLV cores** A) Representative immunoblot probed for MLV capsid
390 protein (p30) in viral pellets in the presence and absence of co-factors (indicated below the blot). The
391 concentration of melittin is 12.5 μ g/ml and the small molecules are used at a concentration of 80 μ M. B)
392 The bar graph shows the quantification of the percentage p30^{CA} recovery after the addition of co-
393 factors. The graph represents the mean \pm SD from three independent experiments. Statistical
394 significance is analyzed using one-way ANOVA. P values are indicated by *, ***P<0.001, **P<0.01, ns:
395 not significant. C) Electron micrographs of MLV before and after treatment with co-factors (indicated

396 below each micrograph). The green arrowhead points to mature MLV particles, the orange arrowhead
397 points to immature particles, and the white arrowhead points to intact MLV cores.

398

399 **Figure 3: MLV packages IP5/6:** The bar graph shows the absolute amount in pmole/ μ l of A) IP6 and B)
400 IP5 packaged in HIV-1 and MLV. Mock represents supernatant from cells transfected with empty vector
401 control. The amounts of C) IP6 and D) IP5 in HIV-1 and MLV are normalized to the respective capsid
402 protein levels in each virus. The graphs represent the mean \pm SD of four independent experiments.
403 Statistical significance is analyzed using Student's t-test. P values are indicated by *, ** $P < 0.01$, * $P < 0.05$,
404 ns: not significant.

405

406 **Figure 4: MLV R3 capsid mutants package IP6/5 but are non-infectious** The bar graphs depict the
407 packaging of A) IP6 and B) IP5 within WT, R3K, and R3A MLV viruses. The amount of IP6 and IP5 is
408 normalized to the amount of capsid protein in each of the virus preparations. The graphs represent the
409 mean \pm SD from two independent experiments. C) A representative immunoblot against p30^{CA} in the
410 supernatant and cell lysates of cells transfected with either WT MLV or R3 capsid mutants of MLV (R3A
411 and R3K). β -actin is used as a loading control. D) WT, R3A, and R3K MLV plasmids were co-transfected
412 with pBabe-Luc into HEK 293T cells. Viruses produced by these transfected cells were used to infect
413 HT1080mCAT cells, and lysates of these cells were assayed for luciferase activity. Specific infectivity was
414 calculated by normalizing the luciferase values to the MLV p30^{CA} levels (RLU/p30) indicative of the
415 amount of virus in culture supernatants. The graphs represent the mean \pm SD of three independent
416 experiments. Statistical significance is analyzed using one-way ANOVA. P values are indicated by *,
417 **** $P < 0.0001$, ns: not significant.

418

419 **Figure 5: MLV R3 capsid mutants do not respond to IP6 *in vitro*** A) The bar graph indicates the
420 formation of ERT products in WT MLV and R3 capsid mutants of MLV with or without IP6 as indicated in
421 the x-axis of the graph. The y-axis represents absolute copies of luciferase DNA reverse transcribed from
422 the MLV-derived luciferase vector RNA packaged in MLV normalized to the amount of virus in each
423 sample. B) Shown here is a representative immunoblot that was probed for MLV p30^{CA} in viral pellets
424 derived from WT, R3K, and R3A MLV, under conditions with or without co-factors, as indicated above
425 the blot. The concentration of melittin used was 12.5 μ g/ml, and co-factors (IP6 and Inositol) were used
426 at 80 μ M. C) The bar graph shows the percentage p30^{CA} recovery in pellets after lysis of WT, R3A, and
427 R3K virions in the presence of the indicated co-factors. The graphs represent the mean \pm SD from three
428 independent experiments. Statistical significance is analyzed using two-way ANOVA. P values are
429 indicated by *, **** $P < 0.0001$, ns: not significant.

430

431 **Figure 6: Cellular IP6/IP5 levels contribute to MLV infectivity.** A) Control, IPMK-KO, and IPPK-KO cells
432 were transfected with a WT MLV plasmid. The virus was collected from these cultures 48 hours after
433 transfection and both supernatants and cell lysates were analyzed by immunoblotting against p30^{CA}. β -
434 actin is used as a loading control. B) Virus release efficiency in the transfected cells. C) Control cells,

435 IPMK-KO cells, and IPPK-KO cells were transfected with viral plasmids. Supernatants from these
436 transfected cells were then used to infect control cells and these cells were then assayed for luciferase
437 activity. Specific infectivity was calculated by normalizing the luciferase values to the MLV p30^{CA} levels
438 (RLU/ p30^{CA}). D) Control cells were transfected with Env-defective MLV plasmid (MLV Gag-Pol),
439 Xenotropic MLV Env plasmid, and pBabe-Luc plasmid. Viruses produced by these transfected cells were
440 then used to infect control, IPMK-KO, and IPPK-KO cells and these cells were then lysed and assayed for
441 luciferase activity. Infectivity was calculated by normalizing the luciferase values to the actin levels
442 (RLU/Actin) in the target cells to correct for differences in cell density between control and KO cells. E)
443 Control cells were transfected with HIV-1 pNL4.3ΔEnv (which carries Luciferase in place of the *env* gene)
444 and with the MLV Xenotropic envelope plasmid. Virus from these transfected cells was then used to
445 infect control, IPMK-KO, and IPPK-KO cells and these cells were then lysed and assayed for luciferase
446 activity. Infectivity (RLU/Actin) was measured in control and KO cell lines. The graphs represent the
447 mean ± SD of two independent experiments with two technical replicates in each experiment (n=4).
448 Statistical significance is analyzed using one-way ANOVA. P values are indicated by *, **** P<0.0001,
449 ***P<0.001, *P<0.05, ns: not significant. F) Control and KO cells were transfected with Env-defective
450 MLV plasmid (MLV Gag-Pol), Xenotropic MLV Env plasmid, and pBabe-Luc plasmid with or without IPMK
451 or IPPK expression plasmids. Viruses produced by these transfected cells were then used to infect
452 control cells and these cells were lysed and assayed for luciferase activity. The bar graph shows the
453 rescue of MLV infectivity in KO cells when the missing kinases in the KO cells were added in trans. G)
454 Control cells were transfected with Env-defective MLV plasmid (MLV Gag-Pol), Xenotropic MLV Env
455 plasmid, and pBabe-Luc plasmid. Control and KO cells were then transfected with the MINPP1
456 expression plasmid or a control plasmid and infected with the virus from the other transfected culture.
457 The cells were lysed and assayed for luciferase activity. The bar graph shows MLV infectivity in control
458 and KO cells transfected with 600 ng of either the empty vector pcDNA3.1 or the MINPP1 expression
459 plasmid. The graphs represent the mean ± SD of two independent experiments with two technical
460 replicates in each experiment (n=4). Statistical significance is analyzed using two-way ANOVA. P values
461 are indicated by *, **** P<0.0001, ** P<0.005, *P<0.05, ns: not significant.

462

463 **Figure 7: IP6/5 is required for reverse transcription during MLV infection** Percentage MLV DNA
464 synthesis measured in infected cells (control and IP-KO cells) using qPCR with primers for A) Early B)
465 Intermediate and C) late reverse transcription products. Before infection, virus preparations were
466 treated with DNase to remove plasmid DNA, as described in Materials and Methods. DNA was extracted
467 from cell lysates at indicated time points within the first 10 hours after infection. D) As some of the viral
468 particles contained pBabeLuc-derived RNA, DNA synthesis was also measured using luciferase primers as
469 in ERT assays. DNA copies of MLV and luciferase were normalized to host CCR5 copies to account for
470 differences in cell density; in each graph, “100” represents the value obtained at 10 hours after infection
471 in the control cells. The graphs represent the mean ± SD of two independent experiments with two
472 technical replicates in each experiment (n=4). Statistical significance is analyzed using two-way ANOVA.
473 P values are indicated by *, **** P<0.0001, ***P<0.001, ** P<0.005, *P<0.05, ns: not significant.

474

475

476

477 **STAR METHODS**

478 **Cells and viruses**

479 *MLV production for ERT assays*

480 All cell lines used in this study were maintained in Dulbecco's modified Eagle's medium (DMEM)
481 supplemented with 10% fetal bovine serum (Hyclone), 100 U/ml of penicillin, and 100 µg/ml of
482 streptomycin, and were grown at 37°C with 5% CO₂. MLV for ERT assays was produced from HEK293T
483 cells by co-transfecting 5µg of pNCS plasmid (a full-length MLV genome, a kind gift from Stephen Goff,
484 ³¹), and 5µg pBabe-Luc plasmid (a pBabe-puro-derived luciferase vector ³²) in a 10cm dish using TransIT
485 293 (Mirus Bio). (Transfecting these plasmids together will give rise to virions with WT MLV proteins;
486 some will contain the full-length, replication-competent MLV genome and some will contain the
487 pBabeLuc genome.) Medium was changed ~18h post-transfection. Virus-containing supernatant was
488 collected 48h post-transfection, pooled from several plates, and cleared by filtration through a 0.45µ
489 filter. The filtered virus-containing supernatant was pelleted through a 20% (w/v) sucrose cushion,
490 prepared in 1X HS buffer (10mM Hepes, pH 7.4, 140mM NaCl) in an SW28 rotor (Beckman) at 25000 rpm
491 for 2 hours. The viral pellet in each tube was resuspended in 150µl of 1X HS buffer and stored at -80°C
492 for use in ERT assays (ERT virus). The amount of p30^{CA} in the pellet was quantitated using p30 ELISA (Cell
493 Biolabs) according to the manufacturer's protocol.

494 *MLV production for infectivity assays*

495 For infectivity assays, MLV was produced from three different cell lines 1) control cells, which are
496 HEK293T cells; 2) IPMK-KO HEK293T cells, where the IPMK gene had been knocked out ²⁴; and 3) IPPK-
497 KO HEK293T cells, where the IPPK gene had been knocked out ²⁴. All three cell lines were a gift from Leo
498 James. MLV was produced in control cells and the two KO cells in parallel, by co-transfecting 5µg of Env-
499 defective MLV plasmid (which also encodes Glyco-Gag ³³) (MLV Gag-Pol), 1 µg of Xenotropic MLV Env
500 plasmid (a kind gift from Henrich Gottlinger ³⁴), and 5µg of pBabe-Luc in a 10 cm dish. The R3 MLV capsid
501 mutant viruses (R3K and R3A) were produced from HEK293T cell line by co-transfecting 5µg of pNCS
502 plasmid containing the intended mutation and 5µg pBabeLuc plasmid in a 10cm dish using TransIT 293
503 (Mirus Bio). Medium was changed ~18 hours post-transfection. Virus-containing supernatant was
504 collected 48h post-transfection and cleared by filtration through a 0.22µ syringe filter. Filtered
505 supernatants were stored at -80°C.

506 **Reverse transcription assays**

507 To assess ERT, 100 µl of ERT virus aliquots were first digested with DNase I (TURBO DNA-free™ Kit,
508 ThermoFisher Scientific) to remove transfected DNA contamination. DNase I digestion was carried out in
509 a total volume of 200µl at 37°C for 30 mins followed by treatment with DNase I inactivation resin
510 supplied with the kit according to the manufacturer's instructions. The treated virus was then used for
511 ERT assay. Each ERT reaction was set up in a total volume of 20µl containing 25mM Tris pH 7.4, 75mM
512 NaCl, 60µM dCTP, 46µM dGTP, 80µM dTTP, 52µM dATP, 0.18mM rCTP, 1.75mM rGTP, 0.7mM rUTP,
513 6.7mM rATP, 3.3 mM MgCl₂, 40 µM IP6, 6.25 µg/ml melittin (Sigma-Aldrich) and 8 µl of DNase-treated
514 ERT-virus unless otherwise specified. The concentrations of Tris, rNTPs, and MgCl₂ were similar to those
515 used in Christensen et al. ¹¹, while the dNTP concentrations were 10-fold higher than those in
516 Christensen et al. ¹¹. The reaction was incubated at 37°C for 10 hours for the synthesis of reverse-

517 transcribed DNA products. DNA products were column-purified using Nucleospin Gel and PCR clean-up
518 kit (Machery-Nagel) and eluted in 25ul of elution buffer (Machery-Nagel). The reverse-transcribed
519 luciferase DNA was quantitated using SYBR Green 1-based qPCR with FastStart Essential DNA Green
520 Master (Roche Life Sciences) and primers luc PB F and luc PB R (Key Resources Table) that targeted the
521 luciferase coding sequence. For measuring ERT kinetics, the ERT reaction was halted at time points
522 specified in the figures: the reaction mixture was immediately applied to the column for purification of
523 the products and quantification using qPCR. Primers used for the quantification were either specific to (-
524) strand strong stop (MSSF4, MSSR2,³⁵) for early products; sequences synthesized after the first-strand
525 transfer (MFST-F, MFST-R) for intermediate products; or those made after second-strand transfer for
526 late products (M2ST-F, M2ST-R). The target sequences for these primers are present in both pNCS (full-
527 length MLV genome) and pBabe-Luc to enable the detection of the total amount of DNA products
528 formed from both MLV RNA and pBabe-Luc RNA. Quantification of copies from qPCR was done using a
529 standard curve generated with every assay using serial dilutions of pBabeLuc plasmid. Ct values of test
530 samples were within the range of the standard curve. To amplify the full luciferase coding sequence
531 (CDS), primers Luc34F and Luc1525R were used³⁶. Sequences of all primers are given in the Key
532 Resources Table.

533 ERT activity for R3 capsid mutants (R3K and R3A) was assessed as above. The ERT products (luciferase
534 DNA) from WT, R3K and R3A measured using qPCR were normalized to the amount of virus, which was
535 quantified using MLV p30 ELISA (Cell Biolabs).

536 To assess whether IP6 affects the enzymatic activity of MLV RT, we measured RT activity in lysed virions
537 using the SG-PERT assay³⁷ as follows. A 50 µl aliquot of ERT virus was treated with 50 µl 2X-PERT lysis
538 buffer containing 0.25% Triton X-100, 50mM KCl, 100mM Tris-Cl pH7.4, and 40% glycerol. These virus
539 lysates were then added to 40 ng of MS2 RNA (Sigma Aldrich), primers (MS2-F and MS2-R), 2X ERT
540 buffer without rNTPs, and different concentrations of IP6, and incubated at 37°C for 50 mins for reverse
541 transcription. Standards were made by serial dilution of recombinant MMLV RT (ThermoFisher
542 Scientific) and incubating with an external template and primers parallel to test samples. Reverse-
543 transcribed products were column-purified and quantitated by SYBR green-based qPCR using primers
544 MS2-F and MS2-R.

545 **Core stability assay**

546 To assess the stability of MLV cores in the presence of small molecules, ERT-virus preparations were
547 used. The standard reaction contained, where indicated, 12.5 µg/ml of melittin; 80 µM of IP6, IS6, HCB,
548 or inositol; and/or rNTPs at 6.7 mM each. The reaction was incubated at 37°C for 1 hour and then
549 centrifuged through a 20% sucrose cushion in an SW 55 Ti rotor (Beckman) at 28000 rpm for 1.5 hours.
550 Pelleted material was resuspended in 100µl 1X NuPAGE LDS sample buffer (Invitrogen) containing
551 NuPAGE sample reducing agent (Invitrogen) and 1X HALT protease inhibitor and analyzed for p30 by
552 immunoblotting.

553 To measure RT activity in the pellets, the pelleted material was resuspended in 100µl of 1X PERT lysis
554 buffer, diluted 10-fold with a buffer comprised of 25 mM KCl + 50 mM Tris HCl pH 7.5, and assayed using
555 the SG-PERT procedure described above.

556 **Electron microscopy**

557 The effects of small molecules upon the stability of viral cores were also analyzed using electron
558 microscopy. After samples were incubated in melittin and small molecules at 37°C for 1 hour as
559 described above, they were fixed by adding 2% glutaraldehyde solution. The fixed samples were then
560 centrifuged in a beam capsule and the pellets were stained, sectioned, and visualized by transmission
561 electron microscopy; details of methods are available on request.

562 **Enzymatic assays of inositol phosphates**

563 IP5 and IP6 were quantified by enzymatic conversion to radiolabeled IP6 and IP7 in reactions catalyzed
564 by purified recombinant Ipk1 and VIP2, respectively, in the presence of ATP- $[\gamma\text{-}^{32}\text{P}]$. The enzymes were
565 expressed in *E. coli* and purified as described³⁸. Products were separated by thin-layer chromatography
566 and detected and quantified by phosphorimager analysis. IP5 reactions were performed in 10- μL
567 volumes containing 50 mM Tris (pH 8.0), 10 mM MgCl_2 , 62 ng/ μL GST-atIpk1, with 10 μCi ATP- $[\gamma\text{-}^{32}\text{P}]$
568 (6,000 Ci/mmol, 10 mCi/mL, PerkinElmer Life Sciences). IP6 assay reactions were performed in 10- μL
569 volumes containing 50 mM Bis-Tris (pH 6.0), 10 mM MgCl_2 , 62 ng/ μL GST-hsVIP2, and 10 μCi of ATP- $[\gamma\text{-}^{32}\text{P}]$.
570 Reactions of standards contained 25 fmol to 0.4 fmol of IP5 (Cayman Chemical) or 0.02 pmol to
571 0.62 pmol IP6 (TCI). Prior to addition to the reactions, virus samples were lysed by addition of Triton X-
572 100 to 0.1% (vol/vol) and heated at 95°C for 10 minutes to dissociate bound inositol phosphates from
573 proteins.

574 Ipk1- and VIP2-catalyzed reactions were incubated at 37°C for 60 min. To stop the reactions, 0.5 μL of 20
575 mg/mL proteinase K was added to each sample and incubated at 56°C for 10 min. The samples were
576 then incubated at 70°C for 10 min to heat-inactivate the proteinase K. 2.5 μL of each sample was applied
577 to polyethyleneimine-cellulose thin layer chromatography (TLC) plates (Millipore Sigma 105579) that
578 were pre-dried in an oven at 60°C for at least an hour. The spots were then allowed to air-dry at room
579 temperature for 10 min and were developed in a TLC tank equilibrated with fresh elution buffer
580 consisting of 1.09 M KH_2PO_4 , 0.72 M K_2HPO_4 , and 2.26 M HCl or 2.5 M HCl for IP5 and IP6 reactions,
581 respectively. Plates were dried at 60°C for 20 min. Plates were exposed to a phosphor storage screen
582 that was subsequently scanned on a FLA7000IP Typhoon phosphorimager (GE Healthcare). Images were
583 quantified using Image Studio Lite (LI-COR Biosciences) and metabolites were quantified by interpolation
584 of nonlinear curve fitting by second-order polynomial using GraphPad Prism 9 from values that fell
585 within the useful range of the standards.

586 **Immunoblotting**

587 For specific infectivity assays, MLV p30^{CA} was quantified directly from the virus-containing supernatant
588 using rabbit polyclonal anti-p30^{CA} antiserum. The supernatant was diluted in NuPAGE LDS sample buffer
589 (Invitrogen) containing NuPAGE sample reducing agent (Invitrogen) and 1X HALT protease inhibitor
590 (ThermoFisher Scientific). The virus-producing cells were also prepared using the same NuPAGE LDS
591 sample buffer cocktail, sonicated for complete lysis, and probed as above. For pelleting-based assays,
592 pelletable material was also assayed using the anti-p30^{CA}. Samples to be assayed were boiled at 90°C for
593 5 mins, loaded onto NuPage 4-12% Bis-Tris PAGE, and electrophoresed until the dye reached the bottom
594 of the gel. Separated proteins were then transferred to Immobilon-FL transfer membranes (Millipore).
595 Membranes were blocked using the Intercept (TBS) blocking buffer from LI-COR. After blocking,
596 membranes were probed overnight at 4°C with the rabbit anti-p30^{CA} antiserum diluted in blocking
597 buffer. Membranes were washed with TBS (20mM Tris, pH 7.0, 500mM NaCl) buffer before incubation
598 at room temperature for 1 hour with IRdye800 donkey anti-rabbit secondary antibody (LI-COR)

599 Biosciences). Membranes were imaged on Odyssey system (LI-COR Biosciences) followed by
600 quantification of the bands using Image Studio Lite (LI-COR Biosciences). Cell lysates were also probed
601 for β -actin using mouse anti- β -actin (Abcepta). For detecting MINPP1 in MINPP1-overexpressing cells,
602 cell lysates were probed with anti-MINPP1 antiserum (Fabgenix). In assays measuring infectivity in
603 target cells the amount of β -actin in cell lysates was also quantified. For quantification of band
604 intensities for p30 and β -actin, a standard curve was prepared using dilutions of a test sample run on the
605 same gel. All sample band intensities fell within the linear range of the standard curve.

606 **Virus Quantitation in IP6 Packaging Assays**

607 Quantification of CA proteins in preparations of HIV-1 particles was performed by immunoblotting of
608 viral lysates in parallel with purified recombinant HIV-1 CA (purified according to Ganser et al. ³⁹) and
609 MLV CA-NC protein (purified as described ⁴⁰). The concentrations of the purified reference proteins
610 were determined spectrophotometrically using extinction coefficients that were calculated according to
611 the method of von Hippel ⁴¹. The concentrations were confirmed by protein assay using the
612 bicinchoninic acid method (BCA Protein Assay kit, ThermoFisher). Values obtained by the two methods
613 agreed to within 5%. Samples were subjected to electrophoresis on 4-20% gradient polyacrylamide gels
614 containing SDS (Genscript). Proteins were transferred electrophoretically to nitrocellulose membrane,
615 and the blots were probed with HIV-1 CA-specific monoclonal antibody 183-H12-5C and goat anti-AKR
616 p30^{CA} polyclonal antiserum obtained from the NCI/BCB Repository located at ViroMed Biosafety
617 Laboratories. Following probing with IR dye-conjugated secondary antibodies, the bands were detected
618 with a LI-COR Odyssey imager and quantified using the instrument software. Values for the viral
619 samples were interpolated from standard curves generated from the signals for the recombinant
620 proteins. The preparations were analyzed in three independent experiments, and the mean values were
621 employed in calculating IP6:CA stoichiometry.

622 **Infectivity assays**

623 In all infections, cells were pre-treated with medium containing DEAE-dextran (20 μ g/ml) for 30 minutes
624 before exposure to the virus-containing supernatant, which had been diluted 10-fold in complete
625 medium. This inoculum was left on the cells for 48 hrs and the cells were then lysed for luciferase
626 assays, as in the manufacturer's instructions (Promega)

627 Control 293T cells were infected with viruses produced from control, IPMK-KO, and IPPK-KO cells to
628 measure specific infectivity. Cells were lysed 48h post-infection and assayed for luciferase activity.
629 Specific infectivity was calculated by normalizing luciferase activity to the amount of the virus. The
630 amount of virus was quantified by immunoblotting for p30 in the filtered virus-containing supernatant.
631 Virus release efficiency was calculated using the formula described in Mallery, et. al. for HIV-1 ²⁴, i.e.,

$$632 \quad (virus\ p30)/(virus\ p30 + cell\ p30 + cell\ Pr65)$$

633 For assessing the effect of IP6/5 depletion in target cells upon MLV replication, infectivity assays were
634 performed in which an equal volume of virus-containing supernatant from the control cells was used to
635 infect control, IPMK-KO cells, and IPPK-KO cells as target cells. Infectivity was assayed by measuring
636 luciferase activity in the cell lysates 48 h post-infection and normalizing the values to β -actin levels in the
637 lysates, as determined by immunoblotting. For rescue experiments, control cells, IPMK-KO cells, and
638 IPPK-KO cells were first transfected with 500 ng of IPMK or IPPK expression plasmid (a kind gift of Leo

639 James). After 24 hours the transfected cells were infected with MLV. Cell lysates were assayed as above.
640 Similarly, the effect of MINPP1 expression upon infection was assessed by transfecting control or KO
641 cells with 600 ng of MINPP1 plasmid; the cells were infected 24 h after transfection and harvested for
642 luciferase and actin assays 48 h after transfection.

643 Infectivity assays for the R3 mutants were performed in the HT1080-mCAT cell line³³ using the same
644 procedure described above.

645 **MLV DNA synthesis in infected cells**

646 The reverse transcription products in MLV-infected cells were assayed as follows. Cells were initially
647 infected (following pre-treatment with DEAE-dextran as described above) with virus that had been
648 DNase digested using DNase I, RNase Free (Invitrogen) to remove plasmid DNA contamination. An
649 aliquot of the DNase I-treated supernatant was heat-inactivated at 68°C for 20 mins. These pre-treated
650 virus-containing supernatants were added to control cells, IPMK-KO cells, and IPPK-KO cells. Cells were
651 lysed 3 hours, 6 hours, and 10 hours post-infection and the DNA was extracted using a QIAamp DNA
652 mini kit (Qiagen). Extracted DNA was quantified using SYBR green-based (FastStart Essential DNA Green
653 Master, Roche Life Sciences) qPCR for early, intermediate and late products using the same primers used
654 for assaying kinetics in ERT assays, or for luciferase DNA using the luciferase primers used in the ERT
655 assay. DNA copies were also measured in cells infected with the heat-inactivated virus after DNaseI
656 treatment to assess the effectiveness of the DNaseI treatment. Luciferase DNA copies in cells infected
657 with heated virus were approximately 100-fold lower than in cells infected with unheated viruses. To
658 account for differences in the recovery of DNA from cells, MLV DNA copy numbers measured with each
659 of the primers were normalized to the copy numbers of the cellular gene CCR5 quantified using primers
660 CCR5-For and CCR5-Rev^{32,42}.

661 **Statistical analysis**

662 The statistical significance of all the data was either analyzed by Student's t-test, one-way, or two-way
663 analysis of variance (ANOVA) using GraphPad Prism 10. The figure legends state the statistical test used
664 in each figure.

665

666 **KEY RESOURCES TABLE**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-p30 ^{CA} antisera (Rabbit)	NIH AIDS Reagent Program	NA
Anti-β-actin (Mouse)	Abcepta	AW5280-U100
Anti-MINPP1 (Rabbit)	Fabgenix	MINPP-101AP
Anti-AKR p30 ^{CA} (Goat)	NCI/BCB Repository (ViroMed Biosafety Laboratories)	NA
Anti-HIV-1 p24 ^{CA} clone 183-H12-5C (Mouse)	Creative Biolabs	MRO-928CQ
Critical Commercial Assays		
MMLV p30 ELISA kit	Cell Biolabs	VPK-156
QIAamp DNA mini kit	Qiagen	51304
FastStart Essential DNA Green Master	Roche Life sciences	06402712001
TURBO DNA-free™ Kit	ThermoFisher Scientific	AM1907

Chemicals, peptides, and recombinant proteins		
Melittin	Sigma Aldrich	M2272-5MG
Inositol hexaphosphate (IP6)	Sigma Aldrich	593648
Inositol pentaphosphate (IP5)	Santa Cruz Biotechnology	sc-221502A
Inositol hexasulfate (IS6)	Santa Cruz Biotechnology	sc-215406
Mellitic acid/ Hexacarboxybenzene (HCB)	Sigma Aldrich	M2705-1G
Oligonucleotides		
Luc PB F: TCTGGATACCGGGAAAACGC	This paper	N/A
Luc PB R: TCAGGCGGTCAACGATGAAG	This paper	N/A
Luc34F: GCGCCATTCTATCCGCTGGAAGAT	Rulli et. al. ³²	N/A
Luc1525R: CGGTTG TTA CTTGACTGGCGACGT	Rulli et. al. ³²	N/A
MSSF4: CGTGTATCCAATAAACCCCTCTTGC	Sanchez-Martinez et. al. ³⁵	N/A
MSSR2: GCTGACGGGTAGTCAATCACTC	Sanchez-Martinez et. al. ³⁵	N/A
MFST-F: CAAGAACAGATGGTCCCCAGA	This paper	N/A
MFST-R: GAACAGAAGCGAGAAGCGAAC	This paper	N/A
M2ST-F: GGGTCTTTCATTTGGGGGCT	This paper	N/A
M2ST-R: CGCAGGCGCATAAAAATCAGT	This paper	N/A
MS2-F: TCCTGCTCAACTTCCTGTCTGAG	Vermeire et. al. ³⁷	N/A
MS2-R: CACAGGTCAAACCTCCTAGGAATG	Vermeire et. al. ³⁷	N/A
CCR5-Fwd: CCAGAAGAGCTGAGACATCCG	Thomas et. al. ⁴²	N/A
CCR5-Rev: GCCAAGCAGCTGAGAGGTTACT	Thomas et. al. ⁴²	N/A
Experimental models: Cell lines		
Control cells	Leo James	24
IPMK-KO cells	Leo James	24
IPPK-KO cells	Leo James	24
HEK293T	ATCC	N/A
HT1080-mCAT	³³	
Recombinant DNA		
pNCS	Stephen Goff	N/A
pBabe-Luc	³²	N/A
MLV Gag-Pol	³³	N/A
Xenotropic Env	Heinrich Gottlinger	N/A
pNL4-3 HIV-1	NIH AIDS Reagent Program	N/A
IPMK	Eric Freed	N/A
IPPK	Eric Freed	N/A
MINPP1	Eric Freed	N/A
Software and algorithms		
Graph Pad Prism 10	Dotmatics	N/A
CFX Maestro	Bio-Rad	N/A
ImageStudio Lite	LI-COR Bioscience	N/A

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670

671 **SUPPLEMENTAL INFORMATION TITLES AND LEGENDS**

672 **Figure S1: Optimization of ERT reaction:** A) The bar graph indicates ERT product formation in the
673 presence of different concentrations of melittin (x-axis). B) ERT product formation was also observed
674 when Triton-X was used instead of melittin. IP6 concentration in these assays was 40 μ M. C) Early,
675 intermediate, and late ERT product formation measured during different time intervals (x-axis). D) The
676 bar graph shows the formation of ERT products in different reaction conditions indicated below the
677 graph. Concentration of the rNTP mixture is the same as in the ERT buffer (described in materials
678 methods) unless mentioned otherwise in the X-axis. The label “6.7mM” above three bars refers to the
679 concentration of each rNTP. E) IP6 titration with and without rNTPs in the ERT reaction buffer. The
680 graphs represent the mean \pm SD of three replicates in the qPCR measurement of a single experiment. F)
681 The bar graph shows MLV RT activity measured using SG-PERT assay. Viruses were lysed in PERT lysis
682 buffer containing 0.125% of triton-X for near-complete lysis. Lysed samples were incubated with an
683 external template (MS2 RNA) and primers. Reverse transcribed products were measured using qPCR.
684 The graphs represent the mean \pm SD of three replicates in the qPCR measurement of a single
685 experiment. Except for Figure S1E, all other figures are representative of a single experiment selected
686 from two independent experiments.

687

688 **Figure S2: Pelleting-based stability assays:** A) Immunoblot showing recovery of MLV capsid protein with
689 increasing concentration of IP6. Inositol is used as a negative control at a concentration of 80 μ M. B)
690 Quantification of the p30^{CA} recovery from the immunoblot. C) Immunoblot showing MLV capsid protein
691 (p30^{CA}) recovery after the addition of rNTPs (6.7mM each). IP6 was used as a positive control and
692 inositol was used as a negative control, both at 80 μ M. D) Quantification of percent p30^{CA} recovery from
693 immunoblots of three independent experiments. The graphs represent the mean \pm SD of three
694 independent experiments. Statistical significance is analyzed using one-way ANOVA. P values are
695 indicated by *, ***P<0.001, ** P<0.01, *P<0.05, ns: not significant.

696

697 **Figure S3: R3 residue in MLV capsid is conserved and MLV-WT and R3 capsid mutant particles have**
698 **similar morphology:** A) Sequence alignment of various gammaretrovirus capsid regions shows complete
699 conservation of the R3 residue, which is highlighted in red. The following gammaretroviruses are
700 included in the alignment: Moloney murine leukemia virus (MMLV, Acc No. P03355), Friend virus (FV,
701 Acc No. P26808.2), Amphotropic murine leukemia virus (A-MLV, Acc No. AAO61195.1), Xenotropic
702 murine leukemia virus (XMRV, Acc No. A1Z651.1), Moloney murine leukemia virus neuropathogenic
703 variant (MLVMN, Acc No. Q8UN02.2), endogenous ecotropic MLV in AKR mice (AKV, Acc No. P03356.3),
704 Feline leukemia virus (FeLV, Acc No. NP_047255.1), Koala retrovirus (KoRV, Acc No. Q9TTC1.2), Porcine
705 endogenous retrovirus (PERV, Acc No. CAB65339.1), Gibbon ape leukemia virus (GaLV, Acc No.
706 P21414.2), and RD114 retrovirus (a cat endogenous virus) from a human tumor cell line RD, Acc No.
707 BAM17305.1. Conservation annotation is as follows: an asterisk (*) denotes positions with a single, fully
708 conserved residue, a colon (:) indicates conservation among groups of strongly similar properties, and a
709 period (.) signifies conservation among groups of weakly similar properties. B) Transmission electron
710 microscopy (TEM) analysis of WT and R3 capsid mutants of MLV. HEK293T cells were transfected with
711 full-length MLV-WT or R3A and R3K mutants for virus production. Two days later they were processed
712 for thin-section TEM. The green arrowhead indicates MLV particles displaying electron density

713 underlying the virion membrane, while the red arrowhead points to MLV particles exhibiting electron
714 density in the center of the virion. The scale bar is 200nm.

715

716 Figure S4: **IP6/5 analysis in IPMK-KO and IPPK-KO cells:** A) TiO₂ PAGE showing levels of IP6 in control,
717 IPMK-KO, and IPPK-KO cells. B) The bar graph shows the amount of IP6 and IP5 quantitated from TLC in
718 control, IPMK-KO, and IPPK-KO cells.

719

720 Figure S5: **IP6 is required for MLV replication:** Quantitation of MLV p30^{CA} in the supernatant (A); MLV
721 p30^{CA} (B), and Pr65 (C) in the cell lysates of control cells and KO cells shown in the representative
722 immunoblot of Figure 5A. Infectivity (RLU/Actin) in control cells vs KO cells with viruses produced from
723 D) IPMK-KO and E) IPPK-KO. Infectivity was calculated after normalizing the luciferase values to the actin
724 levels in the target cells to account for differences in cell density between the control and KO cells. The
725 graphs represent the mean ± SD of two independent experiments with two technical replicates in each
726 experiment (n=4). Statistical significance is analyzed using one-way ANOVA. P values are indicated by *,
727 **** P<0.0001, ***P<0.001.

728

729 Figure S6: **MINPP1 expression in control and IP-KO cells:** Immunoblot showing MINPP1 expression in
730 the control and the KO cell lines transfected with 200 ng or 600 ng of MINPP1-expressing plasmid or of
731 pcDNA3.1.

732

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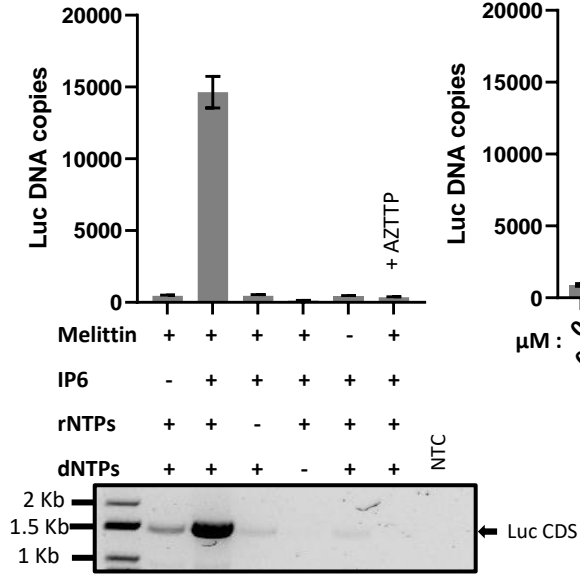
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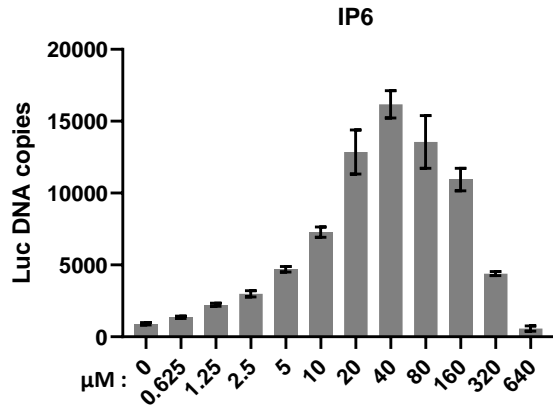
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Figure 1

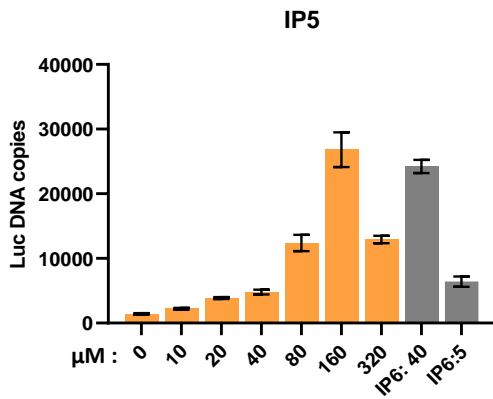
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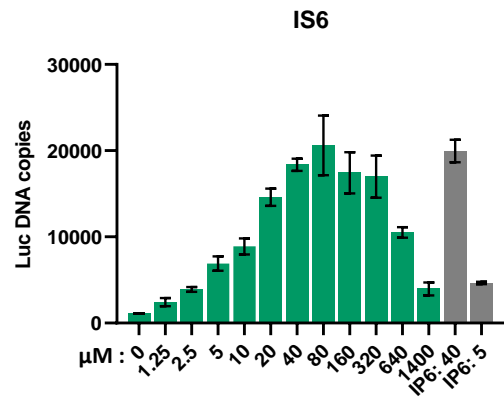
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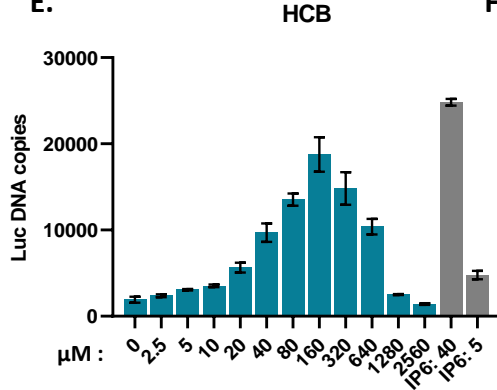
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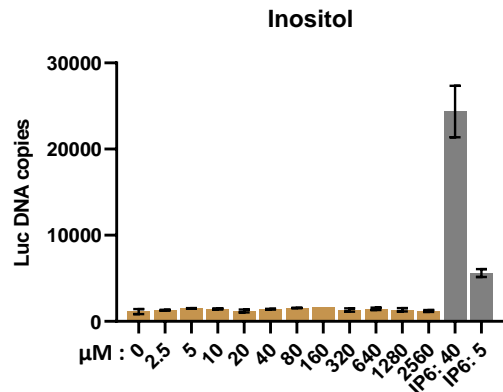
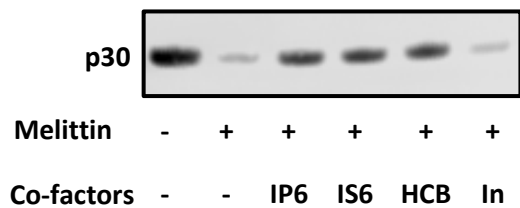
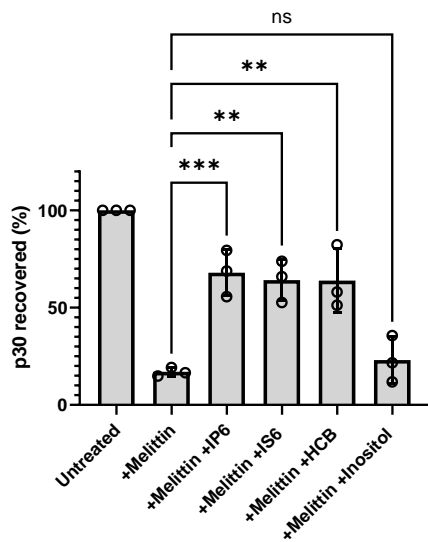


Figure 2

A.

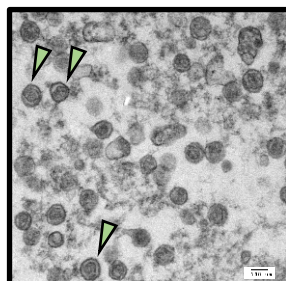


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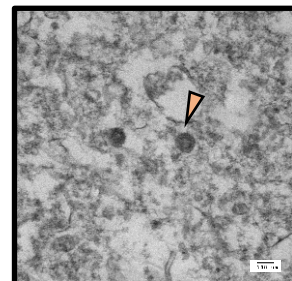


C.

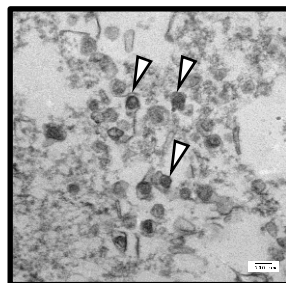
Mature particles
 Immature particles
 Cores



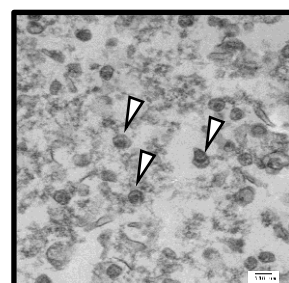
Untreated



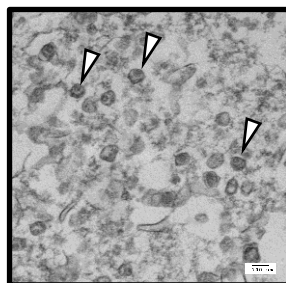
Melittin only



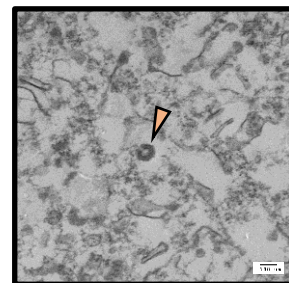
Melittin + IP6



Melittin+IS6



Melittin + HCB



Melittin + Inositol

Figure 3

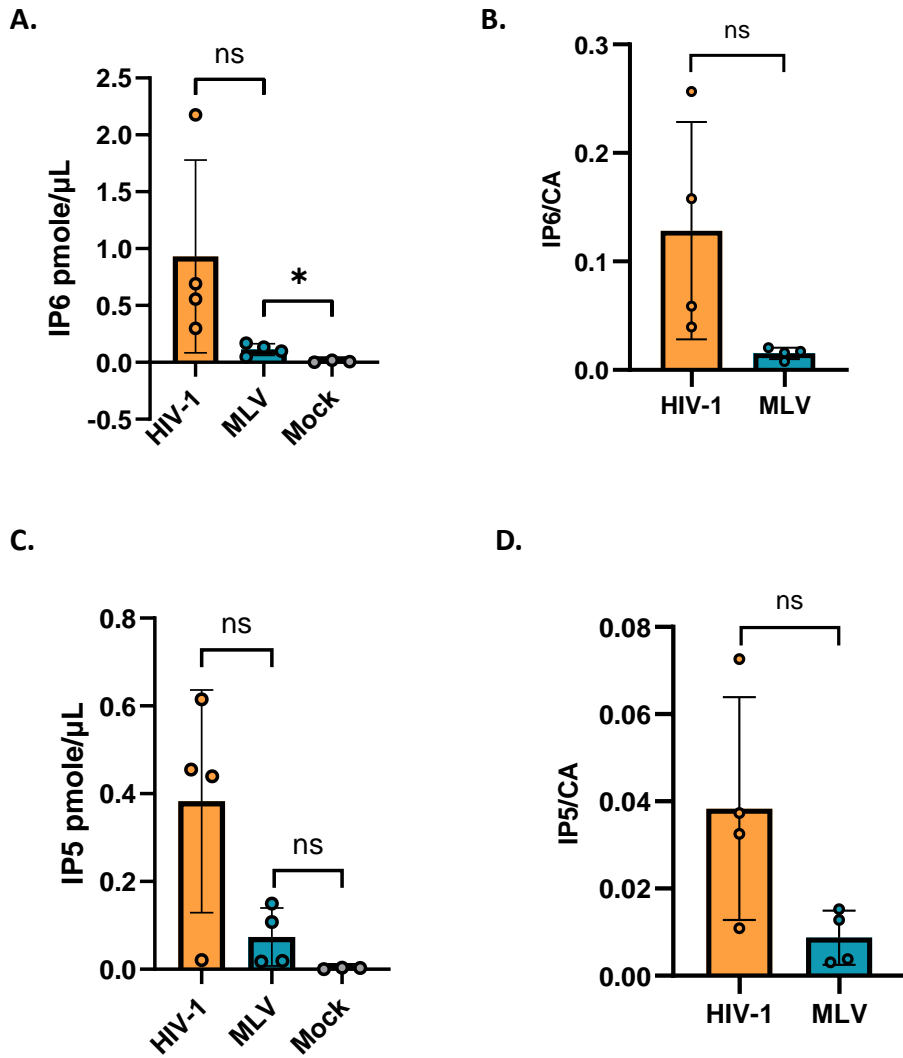
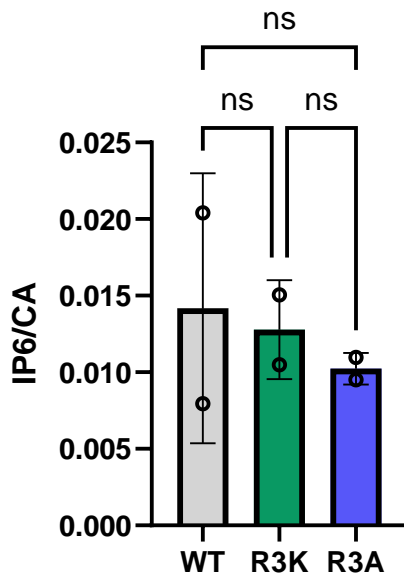
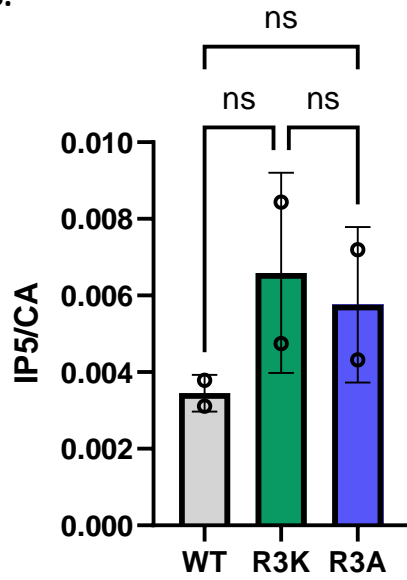


Figure 4

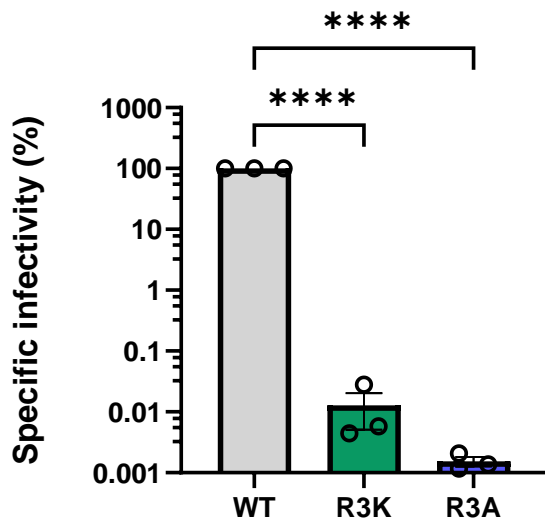
A.



B.



C.



D.

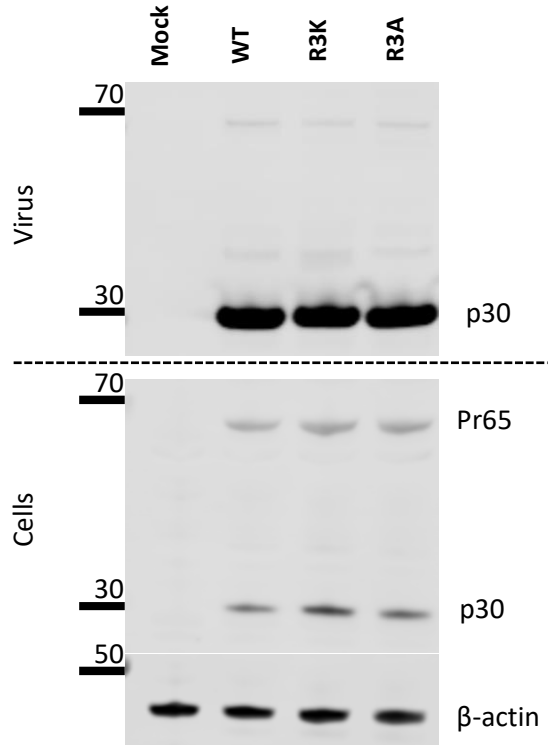


Figure 5

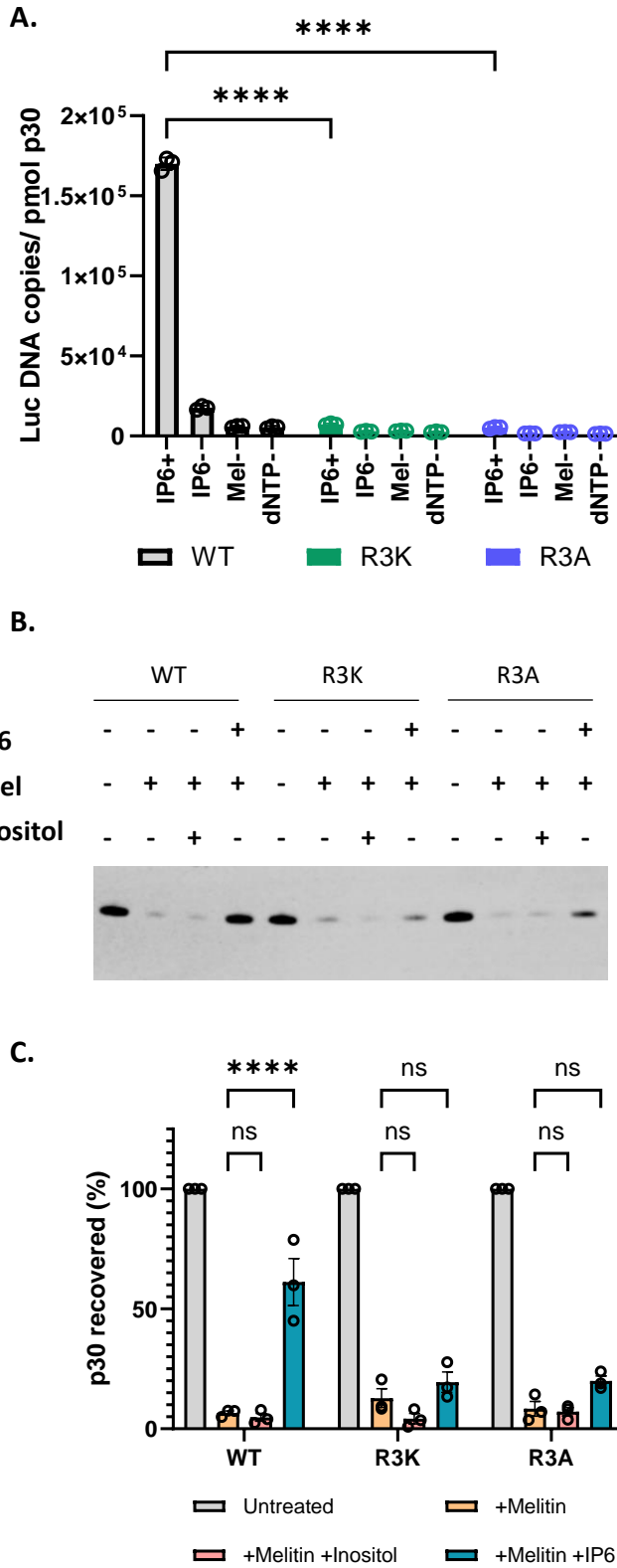


Figure 6

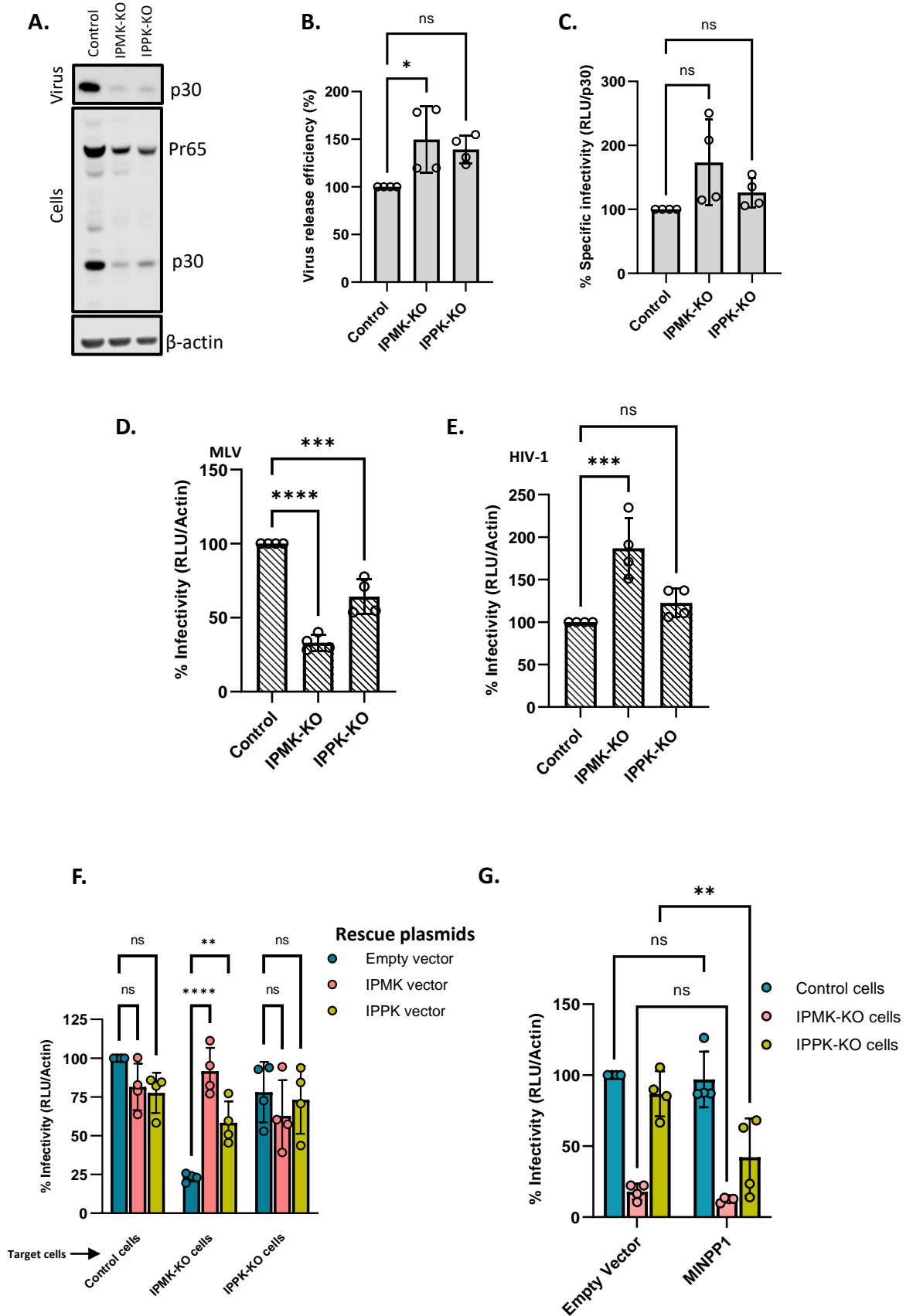
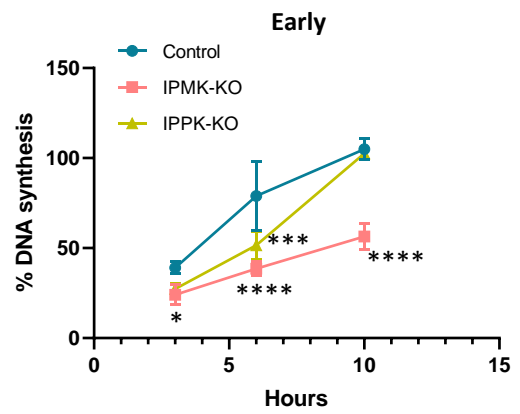
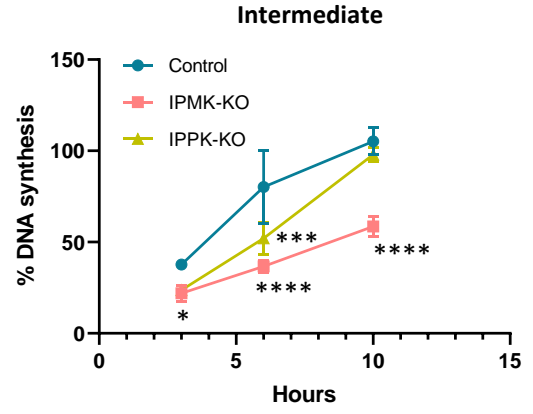


Figure 7

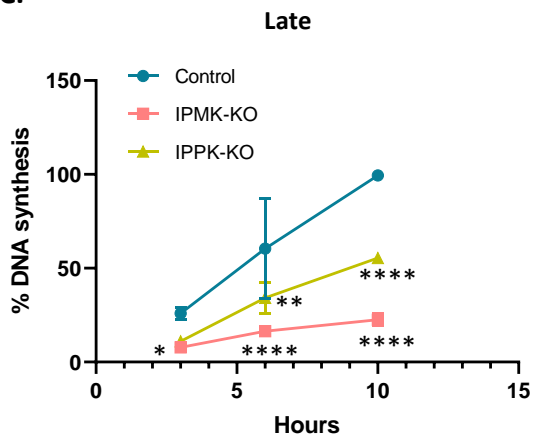
A.



B.



C.



D.

