

1 **Title: An infectious Rous Sarcoma Virus Gag mutant that is defective in nuclear cycling**

2 Running Title: Infectious nuclear cycling defective RSV Gag mutant

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## 18 **Abstract**

19           During retroviral replication, unspliced viral genomic RNA (gRNA) must escape the  
20 nucleus for translation into viral proteins and packaging into virions. “Complex” retroviruses  
21 such as Human Immunodeficiency Virus (HIV) use cis-acting elements on the unspliced gRNA  
22 in conjunction with trans-acting viral proteins to facilitate this escape. “Simple” retroviruses  
23 such as Mason-Pfizer Monkey Virus (MPMV) and Murine Leukemia Virus (MLV) exclusively  
24 use cis-acting elements on the gRNA in conjunction with host nuclear export proteins for nuclear  
25 escape. Uniquely, the simple retrovirus Rous Sarcoma Virus (RSV) has a Gag structural protein  
26 that cycles through the nucleus prior to plasma membrane binding. This trafficking has been  
27 implicated in facilitating gRNA nuclear export and is thought to be a required mechanism.  
28 Previously described mutants that abolish nuclear cycling displayed enhanced plasma membrane  
29 binding, enhanced virion release, and a significant loss in genome incorporation resulting in loss  
30 of infectivity. Here, we describe a nuclear cycling deficient RSV Gag mutant that has similar  
31 plasma membrane binding and genome incorporation to WT virus and surprisingly, is replication  
32 competent albeit with a slower rate of spread compared to WT. This mutant suggests that RSV  
33 Gag nuclear cycling is not strictly required for RSV replication.

34

## 35 **Importance**

36           While mechanisms for retroviral Gag assembly at the plasma membrane are beginning to  
37 be characterized, characterization of intermediate trafficking locales remain elusive. This is in  
38 part due to the difficulty of tracking individual proteins from translation to plasma membrane  
39 binding. RSV Gag nuclear cycling is a unique phenotype that may provide comparative insight

40 to viral trafficking evolution and may present a model intermediate to cis- and trans-acting  
41 mechanisms for gRNA export.

42

### 43 **Introduction**

44 Retroviruses hijack a multitude of host processes to overcome barriers throughout the  
45 viral lifecycle. One such barrier is the nuclear membrane, which protects host genetic data and  
46 allows for regulation of genes by keeping unspliced RNA transcripts from exiting the nucleus.  
47 Retroviral genomic RNA (gRNA) consists of a long unspliced transcript that must escape the  
48 nucleus and traffic to the plasma membrane for virion packaging. Retroviruses, such as HIV,  
49 have evolved trans-acting viral proteins to facilitate the active transport of unspliced gRNA out  
50 of the nucleus via interaction with cis-acting elements on the gRNA. An Arg-rich nuclear  
51 localization signal (NLS) on the Rev protein of HIV allows nuclear entry of non-gRNA-bound  
52 Rev via importin- $\beta$  (1, 2). A nuclear export signal (NES) on Rev allows nuclear export of  
53 gRNA-bound Rev via the exportin Chromosomal Maintenance 1 (CRM1) (1, 3, 4). "Simpler"  
54 retroviruses such as Mason-Pfizer Monkey Virus (MPMV) and Murine Leukemia Virus (MLV)  
55 exclusively use gRNA cis-acting elements in conjunction with host cell export factors (5–8). In  
56 the case of the alpharetrovirus, Rous Sarcoma Virus (RSV), evidence has implicated the Gag  
57 structural protein nuclear cycling as a trans-acting mechanism for exporting gRNA (reviewed in  
58 (9–11)).

59 Initial gross truncation of RSV to study plasma membrane binding unexpectedly found  
60 that a Matrix (MA)-GFP fusion protein was enriched in the nucleus (12). Since RSV-Gag-GFP  
61 with Protease (PR) deleted (hereafter referred to as RSV-Gag<sup>WT</sup>) expresses in the  
62 cytoplasm/plasma membrane at steady state, this pointed toward the full Gag protein potentially

63 trafficking to intermediate subcellular locales (12). To determine whether the non-nuclear  
64 phenotype of Gag was due to size exclusion or nuclear export, Leptomycin B (LMB) was used to  
65 block the CRM1 export pathway of cells transfected with RSV-Gag<sup>WT</sup> (12). With LMB  
66 treatment, RSV-Gag<sup>WT</sup> was shown to rapidly shift to an almost exclusive nuclear expression  
67 (12). Truncation of RSV-Gag<sup>WT</sup> and amino acid manipulation demonstrated that the NES was  
68 located in the p10 domain and that a single (L219A) point mutation blocked nuclear export  
69 resulting in only nuclear expression (12, 13). Further characterization verified NLSs consisting  
70 of a non-canonical importin-11 and transportin-3 (TNPO3) dependent NLS in the tertiary  
71 structure of MA and a canonical four basic amino acid importin- $\alpha/\beta$  dependent NLS motif in  
72 nucleocapsid (NC, K<sub>36</sub>KRK<sub>39</sub>) (12, 14–17).

73         Interestingly, a non-infectious mutant Myr1E that did not accumulate in the nucleus with  
74 LMB treatment exhibited strong plasma membrane binding, increased virion release (1.4xWild  
75 Type, WT), and had a defect in genome packaging (0.4xWT) (12). This suggested a Gag nuclear  
76 localization requirement for genome packaging (12, 18). Myr1E consists of the myristoylated  
77 10-amino acid Src plasma membrane binding domain added to the N-terminal end of RSV-Gag  
78 (12, 19). Another non-infectious mutant, SuperM, consists of RSV-Gag with two Glu swapped  
79 with Lys (E25K and E70K) (20). SuperM also displayed strong plasma membrane binding that  
80 did not accumulate in the nucleus with LMB treatment, increased viral release (3xWT), and had  
81 a severe defect in genome packaging (0.1xWT) (20). To characterize the role of Gag nuclear  
82 localization in genome packaging, a canonical NLS was engineered into Myr1E.NLS (17, 18).  
83 Though both viruses were not infectious; nuclear cycling was enhanced with Myr1E.NLS as  
84 compared to Myr1E, viral release remained the same (1.4xWT), and corresponded to recovered  
85 genome packaging at nearly WT levels (18). To complement this finding, a different study

86 showed that Gag exhibited reduced binding to nuclear import factors importin- $\alpha$  and -11 when  
87 bound to viral RNA containing the  $\psi$  packaging signal (21). In conjunction, Gag bound to  
88  $\psi$ RNA promoted binding to CRM1 (21). Together, these data suggest that RSV requires Gag  
89 nuclear cycling to export unspliced gRNA for subsequent packaging and infectivity.

90 While the previously summarized data suggests Gag involvement with gRNA nuclear  
91 export and packaging, there is also evidence against this argument. There is a gRNA cis-acting  
92 element found in the direct repeats (DR) flanking the *Src* gene of RSV (22, 23). DR deletion and  
93 various DR mutants severely decreased the amount of unspliced RNA transcripts and Gag  
94 protein levels in the cytoplasm of chicken embryo fibroblasts (22, 23). In a gain of function  
95 assay, expression of HIV-1 Gag protein, a proxy for unspliced HIV-1 gRNA translocation into  
96 the cytoplasm, was recovered when RSV DRs were engineered into HIV-1 constructs devoid of  
97 functional Rev protein and Rev response element on the gRNA (22). To parse the mechanism of  
98 nuclear export, the investigators used a three-part assay consisting of RSV constructs encoding  
99 chloramphenicol acetyl transferase (CAT) to report unspliced RNA in the cytoplasm of  
100 transfected cells, fluorescent in situ hybridization (FISH) of reporter viral RNA (vRNA)  
101 containing DR, and dominant-negative mutants of host cellular nuclear export factors Tap and  
102 Dbp5. With this assay, the DR were shown to use and require Tap and Dbp5 to translocate the  
103 viral RNA to the cytoplasm (24). Furthermore, addition of increasing amounts of transfected  
104 RSV Gag did not increase cytoplasmic levels of DR containing CAT reporter vRNA nor did it  
105 increase cytoplasmic levels of  $\psi$  containing CAT reporter vRNA (24). This line of evidence is  
106 corroborated by other studies and mutational analyses which point toward both DR playing a role  
107 in Gag assembly and DR2 as the major cis-acting element for unspliced RNA transport (25–27).

108 These pieces of evidence suggest that RSV Gag nuclear cycling is not involved with or plays a  
109 minor role in gRNA translocation to the cytoplasm.

110 In a survey of various retroviral Gag proteins, our laboratory described a non-nuclear  
111 cycling HIV-MA/RSV-Gag-GFP<sup>ΔPR</sup> (H/RSV-Gag) chimera. Cloning this chimera into a single-  
112 cycle H/RSV<sup>ΔEnv</sup> provirus demonstrated that it produced about half as many infectious particles  
113 as WT virus (28). HIV-MA is myristoylated like the non-infectious Myr1E RSV mutant and  
114 presumably would cause similar genome packaging defects that result in severe infectivity  
115 defects, but this is not the case and suggests additional mechanisms are involved. Alteration of  
116 plasma membrane trafficking and membrane binding has consequences not limited to genome  
117 packaging (29–32). Briefly, non-myristoylated Gag-membrane interactions are mainly due to  
118 electrostatic interactions and altered hydrophobic interactions with acyl chains such as  
119 phosphatidylinositol-4,5-bisphosphate (PI(4,5)P<sub>2</sub>) or cholesterol depletion can reduce plasma  
120 membrane binding (29–32). Separating the effects of plasma membrane binding and/or  
121 trafficking alterations from nuclear cycling would provide fewer confounding interpretations of  
122 nuclear cycling effects on viral assembly. In addition, the separation of the functions in a  
123 replication competent context would provide insight into overall effects of nuclear cycling during  
124 the viral lifecycle. Replication competency of our infectious, non-nuclear cycling H/RSV  
125 chimera, however, could not be tested due to the major splice donor for Env located in the N-  
126 terminus of MA (28, 33). Here, we characterize a replication competent mutant that contains a  
127 minimal amino acid substitution from a polymorphism found in the NC-NLS of RSV strain  
128 JS11C1, is defective in nuclear cycling, and incorporates gRNA at similar levels to WT.

129

130 **Results**

131 *Validation of NLS's and NES in RSV-Gag and determination of the H/RSV-Gag<sup>WT</sup> nuclear*  
132 *cycling block.*

133 We demonstrated previously that replacement of the RSV MA domain with HIV-1 MA  
134 prevents nuclear cycling of RSV Gag. To probe how HIV-MA blocks nuclear cycling in the  
135 H/RSV-Gag<sup>WT</sup> construct, we tested various RSV deletion and HIV-MA mutation/truncation  
136 constructs in the RSV-Gag<sup>WT</sup> and H/RSV-Gag<sup>WT</sup> backbone respectively (Fig 1A). Plasmid  
137 constructs were transfected in parallel into DF1 cells (chicken embryonic fibroblast) and imaged  
138 16-18 hrs post transfection followed by LMB (10 ng/mL) treatment for 1 hr and further imaging.  
139 At steady state, RSV-Gag<sup>WT</sup> and H/RSV-Gag<sup>WT</sup> displayed diffuse and in many instances both  
140 diffuse and punctate cytoplasmic expression of GFP in contrast to the nearly exclusive nuclear  
141 expression of the previously described RSV-Gag<sup>L219A</sup> mutant (Fig 1B) (13, 28). Recapitulating  
142 previous findings, CRM1 inhibition with LMB resulted in accumulation of RSV-Gag<sup>WT</sup> to the  
143 nucleus but not H/RSV-Gag<sup>WT</sup> (Fig 1C) (13, 28). To further validate our system, previously  
144 described mutants, RSV-Gag<sup>ΔMA</sup> and RSV-Gag<sup>ΔNC</sup> (14), in addition to RSV-CANC were tested  
145 for nuclear accumulation (Fig 1B and C). As expected, RSV-Gag<sup>ΔMA</sup> displayed nuclear  
146 accumulation with LMB treatment indicating strong nuclear import via the NC-NLS, followed  
147 by strong export of the Gag protein via the p10-NES. Recapitulating previous findings, RSV-  
148 Gag<sup>ΔNC</sup> expressed in relatively equal amounts in the cytoplasm and nucleus with LMB treatment  
149 (14). This suggests that the NC-NLS is the dominant NLS in RSV Gag. RSV-CANC, lacking  
150 the NES in p10, displayed nearly exclusive nuclear expression with and without LMB (Fig 1B  
151 and C). This indicates the NC-NLS is sufficient to drive nuclear import and that HIV-MA is  
152 somehow able to inhibit nuclear import in H/RSV-Gag<sup>WT</sup>.

153 HIV Gag, unlike RSV, is myristoylated and this modification is required for proper HIV  
154 Gag trafficking and binding to the plasma membrane (30, 34). To probe whether myristoylation  
155 and plasma membrane trafficking potentially counteract the NLS signal, a known myristoylation  
156 defective G2A mutation was tested in the H/RSV-Gag<sup>WT</sup> construct (35). H/RSV-Gag<sup>G2A</sup>  
157 expressed primarily in the cytoplasm with little to no plasma membrane accumulation and no  
158 residual puncta on the cell culture dish that would presumably be budded H/RSV-Gag<sup>G2A</sup> virus  
159 like particles (VLPs) (Fig 1B). Additionally, no change in phenotype was seen with LMB  
160 treatment (Fig 1C). This suggests that membrane binding via PI(4,5)P<sub>2</sub> interaction with  
161 myristoylated Gag is not the determining factor for the blocked nuclear import of H/RSV-Gag<sup>WT</sup>.  
162 A possible explanation is that HIV-MA sterically hinders the NC-NLS, or alternatively, HIV-  
163 MA counteracts the NC-NLS by anchoring the polyprotein to some feature in the cytoplasm  
164 (30).

165 To locate HIV-MA residues important for the cytoplasmic retention of H/RSV-Gag<sup>WT</sup>,  
166 we used previously characterized secondary structural domains to sequentially truncate  $\alpha$ -helices  
167 from the N-terminus of the H/RSV-Gag<sup>WT</sup> backbone, leaving the starting Met followed by the  $\alpha$ -  
168 helix of interest (36). H/RSV-Gag<sup>H2</sup> truncates helix-1 through the first four amino acids of helix-  
169 2 (K30-H33) and contains the remainder of helix-2 through the end of HIV-MA (I34-Y132) (Fig  
170 1A). H/RSV-Gag<sup>H3</sup> truncates helix-1, helix-2, helix-2', and begins at the N-terminus of helix-3  
171 (T55-Y132) (Fig 1A). H/RSV-Gag<sup>H5</sup> truncates helix-1 through -4 and begins at the N-terminus  
172 of helix-5 (D96-Y132) (Fig 1A). All three of the truncations of HIV-1 MA restored nuclear  
173 cycling of H/RSV-Gag<sup>WT</sup> (Fig 1B-C). Interestingly, H/RSV-Gag<sup>H2</sup> and H/RSV-Gag<sup>H3</sup> displayed  
174 varying degrees of nuclear expression even in the absence of LMB (Fig 1B). These data



175 demonstrate that the nuclear retention H/RSV-Gag<sup>WT</sup> requires the presence of helix-1 of HIV-1  
176 MA.

177

178 *An alternative Gag mutant separating plasma membrane trafficking and nuclear cycling.*

179 Non-nuclear cycling H/RSV<sup>ΔEnv</sup> pseudotypes were infectious, causing us to question the  
180 importance and requirement of nuclear cycling during the RSV lifecycle. Previously described  
181 mutants Myr1E and SuperM were presumed to overcome nuclear trafficking by enhancing  
182 targeting to the plasma membrane resulting in the loss of genome incorporation (12, 18, 20).  
183 However, the loss may also result from too rapid of virion escape excluding genome  
184 incorporation. Parsing the mechanism requires separation of plasma membrane trafficking from  
185 nuclear localization. To further probe the dominant NC-NLS, we performed a BLAST database  
186 search for known polymorphisms at the characterized NC-NLS (15). We found one  
187 polymorphism, NC-K36E, in the infectious clone (JS11C1) that was constructed from the  
188 genomic viral sequence isolated from a chicken breed in a study by Cui and colleagues (37).

189 In the previous study by Lochmann and colleagues, the NC-NLS was removed by  
190 changing all four positive charges to Ala (15). This Ala mutant represents a fairly significant  
191 change in peptide composition and may have had unintended phenotypic consequences. Since  
192 the study that identified JS11C1 was focused on general characterization and comparison of  
193 avian leukosis viruses prevalent in indigenous agricultural chickens, specific amino acid  
194 polymorphisms were probably not as carefully scrutinized and single, isolated divergent amino  
195 acids may have arisen from sequencing artifacts. To test the phenotype of the polymorphism in  
196 JS11C1, the NC-K36E mutation was engineered into RSV-Gag<sup>K36E</sup> (Fig 2A). Because NC-  
197 K36E is an opposite charge change, we also engineered the more neutral and more structurally

198 similar NC-K36M into RSV-Gag<sup>K36M</sup>. At steady state, RSV-Gag<sup>WT</sup>, RSV-Gag<sup>K36E</sup>, and RSV-  
199 Gag<sup>K36M</sup> displayed diffuse and in many instances both diffuse and punctate cytoplasmic  
200 expression of GFP (Fig 2B). LMB treatment resulted in nuclear accumulation of RSV-Gag<sup>WT</sup>,  
201 but both RSV-Gag<sup>K36E</sup> and RSV-Gag<sup>K36M</sup> remained cytoplasmic even after one hour of treatment  
202 (Fig 2C). These data demonstrated that the single point mutation found in JS11C1 NC was  
203 sufficient to abolish the nuclear cycling of RSV Gag.

204

205 *RSV<sup>K36M</sup> is replication competent but has a reduced rate of spread compared to RSV<sup>WT</sup>.*

206 Compared to the RSV-Gag<sup>WT</sup>, NC-K36E/M mutations were deficient in nuclear cycling.  
207 To test whether these mutations were tolerated in infectious virus, we engineered NC-K36E/M  
208 into an RSV<sup>ΔEnv</sup> single-cycle construct with two fluorescence reporters (Fig 3A). Briefly,  
209 crimson fluorescence protein is encoded outside of the viral genome, while EGFP is incorporated  
210 into the viral genome. This construct is pseudotyped with Vesicular Stomatitis Virus  
211 glycoprotein (VSV-g) upon transfection. Transfected cells fluoresce both crimson and green;  
212 however, subsequently infected, un-transfected target cells fluoresce only green since virions  
213 from the producer cell only encode for EGFP (Fig 3B). Distribution of fluorescent DF1 cells, as  
214 measured by the ratio of infected to transfected cells, was then determined via flow cytometry  
215 five days post transfection. RSV<sup>ΔEnv,K36M</sup> and RSV<sup>ΔEnv,K36E</sup> were able to infect target cells at  
216 about 50% and 10% the rate of RSV<sup>ΔEnv</sup>, respectively (N=6, p<0.01, pairwise Wilcoxon rank  
217 sum test, Fig 3C).

218 To address the possibility of transfection or pseudotyping artifacts contributing to the  
219 apparent infectiousness of the mutants, NC-K36E/M were engineered into replication competent  
220 RCAS-GFP (RSV<sup>WT</sup>, Fig 4A). RSV<sup>WT</sup>, RSV<sup>K36E</sup>, and RSV<sup>K36M</sup> were then transfected into

221 separate dishes of DF1s. Both RSV<sup>K36E</sup> and RSV<sup>K36M</sup> were able to infect 75% or more cells  
222 within two weeks. To compare kinetics, the virus producing cell-line was co-cultured with  
223 uninfected target cells, starting at 1% of the total cell population, and a portion collected every  
224 other day for flow cytometry. Since RSV<sup>ΔEnv,K36E</sup> displayed more severe defects in the single-  
225 cycle assay and RSV<sup>K36E</sup> spread through tissue culture at a markedly slower rate than RSV<sup>K36M</sup>  
226 in preliminary testing (data not shown), RSV<sup>K36E</sup> was not further tested. RSV<sup>K36M</sup> spread  
227 through tissue culture at a reduced rate compared to RSV<sup>WT</sup>. RSV<sup>WT</sup> spread to 93% of the  
228 culture on day six, while RSV<sup>K36M</sup> only spread to 72% on day six (N=5, p<0.01, pairwise  
229 Wilcoxon rank sum test, Fig 4B). Together, the slowed kinetics of replication competent  
230 RSV<sup>K36M</sup> recapitulates the dysfunction seen in RSV<sup>ΔEnv,K36M</sup>.

231 Lys and other positively charged amino acids in NC contribute to nucleic acid binding,  
232 Gag multimerization, and binding dynamics at the plasma membrane (34, 38–44). To address if  
233 the loss of the Lys in RSV<sup>K36E/M</sup> contributes to dysfunction because of the lost NLS and not  
234 simply to a lost basic residue (42–44), we engineered three other point mutations in NC where an  
235 amino acid with a basic charge was changed to Met (NC-R18M, -R44M, and -K62M) and tested  
236 function as described above. All three mutants displayed reduced spread through tissue culture  
237 compared to RSV<sup>WT</sup> on day six with RSV<sup>R18M</sup> spreading to 60%, RSV<sup>R44M</sup> to 72%, and RSV<sup>K62M</sup>  
238 to 65% (N=4-5, p<0.05, pairwise Wilcoxon rank sum test, Fig 4B). The reduction in spread  
239 with these three mutants was as severe as with RSV<sup>K36M</sup> (N=4-5, NS, pairwise Wilcoxon rank  
240 sum test, Fig 4B) and is consistent with a deficiency in nucleic acid binding in contrast to the  
241 inability to cycle through the nucleus.

242

243 *Replication competent RSV<sup>K36M</sup> virus does not regain the ability to cycle.*

244 Multiple passages through tissue culture inherently increase the potential for rescue  
245 mutants to arise. Mutant phenotypes, such as deficient nuclear cycling, can be rescued through  
246 reversion of the mutation, which in the case of a single point mutation in RSV<sup>K36M</sup> is likely and  
247 could potentially explain the ability of the mutant to infect cells. Additionally, secondary  
248 mutations may arise to counter the phenotype produced from the original mutation.  
249 Furthermore, RSV<sup>K36M</sup> may localize Gag differently in the context of full-length virus since  
250 cellular localization of the RSV-Gag<sup>K36M</sup> mutation was tested in a limited construct consisting of  
251 a truncated Gag-GFP fusion protein. To address the discussed possibilities and address the  
252 overall phenotype of full-length RSV<sup>K36M</sup>, we performed immuno-fluorescence staining against  
253 RSV-CA to visualize Gag localization. Briefly, DF1 cells infected with replication competent  
254 virus from Fig 4 were plated on glass coverslips, treated with or without LMB (10 ng/mL) for 1  
255 hr, and fixed in 5% PFA. Samples were blocked, probed with Rb $\alpha$ RSV-CA, probed with  
256 fluorescently labeled Gt $\alpha$ Rb, and nuclei stained with Hoechst. RSV-CA localization in relation  
257 to Hoechst-stained nuclei was then visualized under confocal-microscopy. At steady state  
258 without LMB treatment, both RSV<sup>WT</sup> and RSV<sup>K36M</sup> was expressed in the cytoplasm with no  
259 Hoechst co-localization (Fig 5A, WT-left and K36M-left columns). After LMB (10 ng/mL)  
260 treatment for 1 hr, RSV<sup>WT</sup> was expressed in the nucleus with Hoechst co-localization and  
261 noticeably less expression in the cytoplasm (Fig 5A, WT-right column). In contrast, close  
262 inspection of LMB treated RSV<sup>K36M</sup> infected cells showed a small amount of Gag nuclear  
263 localization compared to untreated cells; however, the majority of the stain remained  
264 cytoplasmic with no Hoechst co-localization (Fig 5A, K36M-right column). Localization of Gag  
265 in RSV<sup>R18M</sup>, RSV<sup>R44M</sup>, and RSV<sup>K62M</sup> infected cells displayed primarily nuclear accumulation  
266 with a minor deficiency compared to RSV<sup>WT</sup> (Fig 5B). Together, these data point to a lack of a

267 rescued nuclear cycling phenotype in RSV<sup>K36M</sup> and suggest that nuclear cycling is not required  
268 for RSV infectivity.

269

270 *Genomic RNA incorporation in RSV<sup>K36M</sup> virus does not statistically differ from RSV<sup>WT</sup>.*

271 As discussed in the introduction, Gag nuclear cycling was speculated to be the trans-  
272 acting mechanism for guiding unspliced gRNA out of the nucleus for subsequent packaging into  
273 virions (18–21). Infectivity loss of the previously described mutants was attributed to a loss in  
274 gRNA virion incorporation (18, 20, 21). To address whether loss in gRNA virion incorporation  
275 contributed to the decreased rate of spread of RSV<sup>K36M</sup>, we quantified gRNA in virions from four  
276 independent experiments. Briefly, RSV<sup>WT</sup>, RSV<sup>K36M</sup>, RSV<sup>R18M</sup>, RSV<sup>R44M</sup>, and RSV<sup>K62M</sup> infected  
277 as well as uninfected DF1s at the endpoint of the replication competent experiment (Fig 4) were  
278 plated equally and equal amounts of media were collected and concentrated over a 20% sucrose  
279 gradient. Equivalent volumes of concentrated media from the four samples were used in parallel  
280 for immuno-blot against RSV-CA and qPCR.

281 Western blots show that relatively equal amounts of virus were produced from the  
282 infected cells and were collected for each of the mutants compared to WT. Quantified RSV-CA  
283 levels were not statistically different except between RSV<sup>WT</sup> and RSV<sup>K62M</sup> (N=4, p<0.05,  
284 pairwise Wilcoxon rank sum test, Fig 6A). To quantify genome incorporation, we amplified a  
285 172-base pair segment flanking the NC-NLS region in triplicate for each of four of the five  
286 independent samples from Fig 4B. To address potential effects of improper endogenous reverse  
287 transcriptase (RT) extension of genome in the RSV mutants due to improper gRNA binding by  
288 NC, we performed the reverse transcription with exogenous Moloney MLV in addition to the  
289 endogenous RT. We observed a small non-statistically significant decrease in RSV<sup>K36M</sup>,

290 RSV<sup>R18M</sup>, RSV<sup>R44M</sup>, and RSV<sup>K62M</sup> gRNA incorporation compared to RSV<sup>WT</sup> (N=3-4, \* p<0.05,  
291 pairwise Wilcoxon rank sum test, Fig. 6B). The observe non-significant trend may likely need  
292 more sensitive assays to parse. Overall, these data suggest that per mL of media relatively equal  
293 amounts of virus were produced with relatively equal amounts of genome incorporated in them.  
294 Furthermore, these data point toward RSV<sup>K36M</sup> playing a dysfunctional role in a different portion  
295 of the viral life cycle and Gag nuclear cycling serving to enhance efficiency of viral spread.

296

## 297 **Discussion**

298 While the underlying mechanisms of Gag protein membrane binding and subsequent  
299 escape of virions are being established and further refined, intermediate trafficking of Gag  
300 proteins and association with gRNA remains elusive and difficult to test. This, in part, is due to  
301 the difficulty of tracking individual proteins and other viral components from production to  
302 incorporation into virions. When coupled with the multifunctional nature of viral proteins where  
303 the same domain can be involved with many aspects across the whole viral lifecycle, this  
304 problem is compounded (45, 46). For example, retroviral NC is involved with nucleic acid  
305 binding during the late phase by binding and ensuring gRNA incorporation which also results in  
306 boosting Gag multimerization (42, 47, 48). During the early phase, NC binds the tRNA<sup>Lys</sup>  
307 necessary for reverse transcription and aids in protecting gRNA/DNA in transit to the nucleus  
308 (49). In the case of RSV, the Gag protein rapidly cycles into and out of the nucleus via NLSs in  
309 MA and NC and an NES in p10 (9–16). While trafficking of gRNA out of the nucleus for  
310 subsequent virion packaging has been implicated as the function of Gag nuclear cycling (9, 10,  
311 18, 21), additional functions and requirement remained to be determined.

312

313 *Identifying how HIV-MA blocks nuclear cycling of H/RSV-Gag<sup>WT</sup>.*

314           The role of RSV-Gag<sup>WT</sup> nuclear cycling was first questioned upon observation that an  
315 H/RSV-Gag<sup>WT</sup> chimera where RSV-MA is replaced with HIV-MA did not cycle through the  
316 nucleus but remained infectious (28). Previously described RSV non-nuclear cycling mutants  
317 attributed the phenotype to strong plasma membrane targeting overcoming the NLS (12, 20).  
318 The resulting non-infectious mutants had severe reductions in genome incorporation and both the  
319 lack of infectivity and genome was attributed to the lack of nuclear cycling. However, plasma  
320 membrane targeting resulted in increased budding efficiency and may have packaging  
321 consequences for the virus separate from abolished nuclear cycling. By strongly targeting to the  
322 plasma membrane, the virion likely assembles too quickly for proper incorporation of necessary  
323 viral components like genome.

324           We hypothesized that HIV-MA myristoylation provided the more moderate plasma  
325 membrane targeting resulting in the abolished nuclear cycling of H/RSV-Gag<sup>WT</sup>. However,  
326 introducing a known mutation (G2A) that abolishes myristoylation did not recover nuclear  
327 cycling (35). Truncation of HIV-MA in an attempt to identify the reason for abolished nuclear  
328 cycling revealed the first  $\alpha$ -helix and basic residues from the N-terminal end of the second  $\alpha$ -  
329 helix to be important for blocking nuclear cycling. Close examination of the crystal structure of  
330 HIV-MA shows that the first and second  $\alpha$ -helices form a protrusion of basic residues (36).  
331 Charged residues play many roles in cellular and viral functions. The truncation of the basic  
332 residue protrusion likely causes a conformational change that masks the NC-NLS or removes a  
333 strong targeting motif counteracting the NC-NLS of RSV-Gag<sup>WT</sup> (Fig 1B), though more  
334 experiments would be needed to probe the mechanism. H/RSV-Gag<sup>H3</sup> likely displays  
335 cytoplasmic steady state expression without LMB addition due to the removal of acidic residues

336 in helix-2 that mask the basic residues of helix-5 (Fig 1B). Further removal of helix-3 acidic  
337 residues in H/RSV-Gag<sup>H5</sup> likely fully un-masks the basic residues of helix-5, allowing for  
338 stronger steady state cytoplasmic retention without LMB addition (Fig 1B).

339

340 *Separating plasma membrane trafficking from nuclear cycling with a minimal mutation.*

341 A single-cycle chimera is not representative of WT virus, so we sought a minimal RSV  
342 mutant that separated plasma membrane targeting from nuclear cycling. We, thus, redirected our  
343 efforts to the more centrally located NC-NLS, which would theoretically separate plasma  
344 membrane binding from nuclear cycling. A polymorphism at position 36 of NC of RSV strain  
345 JS11C1, is defective in Gag nuclear cycling. Distribution of the polymorphism reconstituted in  
346 RSV-Gag<sup>K36E</sup> as well as the less drastic RSV-Gag<sup>K36M</sup> mutation is diffuse throughout the  
347 cytoplasm, similar to WT virus and in contrast to the strong plasma membrane bound phenotype  
348 of previously described non-nuclear cycling mutants. Furthermore, RSV<sup>K36M</sup> remains replication  
349 competent, albeit with modestly reduced rate of viral spread. Together, these data suggest that  
350 plasma membrane trafficking and binding of RSV<sup>K36M</sup> is similar to RSV<sup>WT</sup>, albeit the defective  
351 nuclear cycling.

352 The reduced rate of spread does not appear to be due to reduced genome incorporation  
353 between RSV<sup>WT</sup> and RSV<sup>K36M</sup>. Basic amino acid residues are important for nucleic acid binding  
354 (42–44). However, single basic amino acid to Met substitutions at other positions of NC neither  
355 affected nuclear cycling nor reduced genome incorporation compared to WT, but reduced overall  
356 rate of spread to the same level as RSV<sup>K36M</sup>. This points toward an undetectable (with the assays  
357 used here) gRNA binding deficiency from the lack of Gag nuclear cycling rather than overall  
358 loss of gRNA packaging.



359           While it could be argued that even small amounts of Gag nuclear localization (as seen in  
360 Fig 5A) is enough to traffic gRNA out of the nucleus, one would also expect a more severe loss  
361 in rate of spread with a mutation in the NLS as compared to basic residue to Met mutations not  
362 in the NLS. The rate of spread is similarly reduced in all NC mutants tested compared to RSV<sup>WT</sup>  
363 (Fig 4B), nuclear localization was not abolished in RSV<sup>R18M</sup>, RSV<sup>R44M</sup>, or RSV<sup>K62M</sup> (Fig 5B),  
364 and genome was incorporated similarly between all mutants (Fig 6B). Together, these pieces of  
365 evidence suggest that the dysfunction in replication lies in nucleic acid binding and not the  
366 ability to cycle through the nucleus.

367

368 *Conclusions.*

369           Here, we show that the distinct nuclear cycling that is characteristic of RSV Gag is not  
370 required for viral infectivity. However, RSV Gag nuclear cycling is an intriguing phenotype  
371 whose function remains elusive. In contrast to “complex” retroviruses like HIV, RSV seems to  
372 rely on cis-acting elements on the genome in conjunction with host cellular components to export  
373 the genome out of the nucleus similar to other “simple” retroviruses. Gag nuclear cycling,  
374 however, boosts rate of viral spread and may be a result of more effective gRNA-virion  
375 assembly. Further investigation to identify function may reveal insights to viral protein  
376 trafficking and host-virus interactions such as the evolution of trans-acting elements for genome  
377 export.

378

## 379 **Materials and Methods**

380 *Plasmid constructs*

381 RSV Gag.3h-GFP (John Wills, Pennsylvania State University) contains full length Gag  
382 with PR removed and fused to GFP and will be referred to as RSV-Gag<sup>WT</sup>. Gag.3h-GFP<sup>L219A</sup>  
383 was previously described and will be referred to as RSV-Gag<sup>L219A</sup> (13). RSV-Gag<sup>ΔMA</sup> was  
384 generated first by PCR amplification of RSV-Gag<sup>WT</sup> between the start of p10 and the p10 FseI  
385 site to engineer an XhoI site and start codon directly upstream of p10. Secondly, we used  
386 restriction digest (NEB restriction enzymes) to remove the region between XhoI upstream of MA  
387 and FseI. Products from these two reactions were ligated via In-Fusion HD Cloning Kit  
388 (Clontech®, 639650). RSV-Gag<sup>ΔNC</sup> (Volker Vogt, Cornell University) is Gag with NC and PR  
389 removed and fused to GFP. RSV-CANC was engineered by PCR amplification of the CA region  
390 of RCAS-GFP (Stephen Hughes, NCI-Frederick) to introduce a SacI site followed by a start  
391 codon at the 5' end leading to the SbfI site in the middle of CA. RSV-Gag<sup>WT</sup> was then digested  
392 with SacI and SbfI and ligated to the amplified piece. RSV-Gag<sup>K36E/M</sup> was engineered into RSV-  
393 Gag<sup>WT</sup> and RSV-Gag<sup>L219A</sup> by restriction digest to remove the region between SbfI at CA and  
394 PspOMI at EGFP with subsequent ligation of PCR products via In-Fusion HD Cloning.

395 H/RSV-Gag<sup>WT</sup> has been previously described (28). Truncation mutants were generated  
396 by PCR amplification of H/RSV-Gag<sup>WT</sup> with a forward primer that started at the SacI restriction  
397 site, spanned the start Met, and bridged to the helix of interest with a reverse primer from the  
398 FseI restriction site. H/RSV-Gag<sup>WT</sup> was then digested with SacI and FseI and products were  
399 ligated via In-Fusion HD Cloning. The resulting construct thus contained the starting Met  
400 followed by the helices of interest.

401 RCAS-GFP was used for replication competent RSV<sup>WT</sup>. RSV<sup>K36E/M</sup> was engineered by  
402 two-step PCR amplification between SbfI at CA and SnaBI at RT with site-directed mutagenesis  
403 of K36, restriction digest to remove the region between the two sites, and ligation of products via

404 In-Fusion HD Cloning. NC-R18M, -R44M, and -K62M mutations were engineered into RSV<sup>WT</sup>  
405 by two-step PCR amplification between SacII in CA and SnaBI in RT with site directed  
406 mutagenesis at the referenced amino acid positions, restriction digest to remove the region  
407 between the two restriction sites, and ligation of products via In-Fusion HD Cloning. RSV<sup>ΔEnv</sup> in  
408 the two-color single-cycle provirus system has been previously described (50). Briefly, Env was  
409 removed from RSV<sup>WT</sup> and was further modified to contain a second reporter—in this case  
410 Crimson—outside of the downstream LTR. RSV<sup>ΔEnv,K36E/M</sup> was generated by restriction digest  
411 of RSV<sup>ΔEnv</sup> and RSV<sup>K36E/M</sup> at SacII at CA and AgeI at IN, and ligation of products. The two-  
412 color reporter system is pseudotyped with VSV-g (NIH AIDS Reagent Program) on a separate  
413 plasmid (51).

414

#### 415 *Cells*

416 The DF1 cell line was obtained (ATCC, CRL-12203) and maintained in Dulbecco's  
417 modified Eagle's medium (DMEM, Sigma, D6429-500ML) supplemented with 7.5% fetal  
418 bovine serum (FBS, Gibco, 10437-028), 1% chicken serum (Sigma, C5405), 2 mM L-glutamine  
419 (Sigma, G7513-100ML), 1 mM sodium pyruvate (Sigma, S8636-100ML), 10 mM minimal  
420 essential medium nonessential amino acids (Sigma, M7145-100ML), and 1% minimal essential  
421 medium vitamins (Sigma, M6895-100ML). DF1s stably infected with RSV<sup>WT</sup> and derived  
422 mutants were similarly maintained.

423

#### 424 *Virus production*

425 RSV VLPs were produced by FuGENE® 6 Transfection Reagent (Promega, E2691)  
426 transfection of DF1s at 50% confluence with 1 μg of viral plasmid. For single-cycle virus, VSV-

427 g was added in a 1:9 ratio. Media containing virus (Viral Media) was collected two days post  
428 transfection by aspiration. Viral media was then frozen at -80°C for a minimum of 1 hr to lyse  
429 cells, thawed in a 37°C water bath, precleared by centrifugation at 3000 x g for 5 min, and  
430 supernatant collected by aspiration. Aliquots were stored at -80°C and subsequently used for  
431 assays. For assays requiring viral concentration, supernatant collected after preclearing was  
432 pelleted through a 100 µL 20% sucrose cushion (20% sucrose, PBS) for 2 h at 30000 x g at 4°C.  
433 Supernatant and sucrose buffer was aspirated off leaving a small amount (~10 µL) of sucrose  
434 buffer so as not to aspirate the viral pellet. Viral pellets were stored at -80°C.

435

#### 436 *Infectivity assays and flow cytometry*

437 For single-cycle infectivity assays, DF1 cells were transfected at 50% confluency in 6-  
438 well format. Five days post transfection, all cells were collected for flow cytometry. For  
439 replication competent infectivity assays, DF1 cells at 50% confluency were transfected in 6-well  
440 format. To remove the variable of both transfected and infected cells in transfected culture, viral  
441 media was collected five days post transfection. Fresh cells were then plated in separate 60 mm  
442 dishes and transduced with 500µL of viral media. Cells were then passaged till the population of  
443 GFP (+) cells approached 100% as determined by flow cytometry. Infected and non-infected  
444 cells were then co-cultured starting at 1% infected. Half of the cell population was collected  
445 every two days for flow cytometry.

446 Cells were collected by washing with PBS and treated with 10 mM TrypLE™ Express  
447 Enzyme (Gibco; Cat. No. 12605028). Lifted cells were then collected with PBS and added to  
448 10% PFA to a final concentration of 5%. After 10–20 min incubation at room temperature, the

449 cells were centrifuged at 300 x g for 5 min, supernatant removed, and 300  $\mu$ L of PBS added.

450 Cells were analyzed for fluorescence using a BD Accuri C6 flow cytometer.

451

#### 452 *Microscopy*

453 Fluorescence microscopy was performed on an Olympus IX70 inverted microscope using

454 a Qimaging Rolera Fast camera and Qimaging software. Cells were first plated on glass bottom

455 dishes (MatTek, P35G-1.5-14-C) and transfected with the afore mentioned plasmids. 16-18 hrs

456 post transfection cells were imaged followed by LMB treatment and subsequent imaging.

457 Images were captured using a 100x oil immersion objective. Gain was adjusted so as not to

458 over-expose cells in focus. Confocal fluorescence microscopy was performed on a Leica TCS

459 SP8 inverted spectral confocal microscope. Cells from the replication competent assay were first

460 plated on glass coverslips in 6-well format. 24 hrs post plating, cells were fixed with 5% PFA.

461 Cells were blocked in 5% goat serum in PBST (10% Tween-20) for 1 hr at room temperature,

462 followed by incubation in RSV-CA antibody raised in rabbit (NCI-Frederick, NCI 8/96) at a

463 1:1000 dilution in blocking buffer. Cells were then washed with PBST (10% Tween-20) 3 x

464 5min, followed by incubation with Gt $\alpha$ Rabbit AlexaFluor 555 at a 1:10000 dilution in blocking

465 buffer. Cells were washed once with PBST and 1 $\mu$ L of Hoechst, followed by 3 x 5 min washes

466 with PBST. Cover slips were then mounted on microscope slides, sealed with clear nail polish,

467 and stored at 4°C. Images were captured using a 100x oil immersion objective and z-stacks were

468 captured as optimized by Leica LASX software.

469

#### 470 *Western Blot*

471 One mL of supernatant collected after preclearing thawed media containing virus was  
472 pelleted via centrifugation through a 20% sucrose cushion (20% sucrose, PBS) for 2 h at 30000 x  
473 g at 4°C. Supernatant and sucrose buffer were aspirated off, leaving a small amount of sucrose  
474 buffer so as not to aspirate the viral pellet (~10 µL). Ten µL of 2x sample buffer (50 mM Tris,  
475 2% sodium dodecyl sulfate [SDS], 20% glycerol, 5% β-mercaptoethanol) was added to pelleted  
476 virus and heated to 95°C for 5 min before loading.

477 Cell samples were washed with PBS and trypsinized with 10 mM EDTA. Cells were  
478 then collected with PBS, centrifuged at 300 x g for 5 min, and supernatant removed. Twenty µL  
479 of RIPA extraction buffer with protease inhibitor was then added to each sample (52). The  
480 samples were then kept on ice and vortexed every 5 min for 20 min, followed by centrifugation  
481 at 10000 rpm for 10 min at 4°C. Supernatant was then transferred to a new tube, 20 µL of 2x  
482 sample buffer added, and heated to 95°C for 5 min before loading.

483 Samples were separated on a 10% SDS-PAGE gel and transferred onto a 0.22 µm pore  
484 size polyvinylidene difluoride (PVDF) membrane. Membranes were blocked for 1 hr at room  
485 temperature with 4% nonfat dry milk in PBST (10% Tween-20). Membranes were then  
486 incubated with anti-RSV-CA antibody raised in rabbit (NCI-Frederick, NCI 8/96) at a 1:500  
487 dilution in blocking solution for 1 hr at room temperature. After blots were washed with PBST  
488 (3 x for 5 min), a goat anti-rabbit peroxidase (HRP)-conjugated secondary antibody (Sigma,  
489 A0545) was applied at 1:10,000 dilution in blocking solution. After 1 hr, blots were again  
490 washed 3x with PBST and imaged. Immobilon Classico Western HRP substrate (Millipore) was  
491 used for visualization of the membranes with a chemiluminescence image analyzer (UVP  
492 BioSpectrum 815 Imaging System).

493

494 *Genome incorporation via qPCR*

495 One mL of supernatant collected after preclearing thawed media containing virus was  
496 pelleted via centrifugation through a 100  $\mu$ L 20% sucrose cushion (20% sucrose, PBS) for 2 h at  
497 30000 x g at 4°C. Supernatant and sucrose buffer were aspirated off, leaving a small amount of  
498 sucrose buffer so as not to aspirate the viral pellet (~10  $\mu$ L). Sample (~5  $\mu$ L) was reverse  
499 transcribed with added Moloney MLV. Virus with 0.5  $\mu$ L each of 100  $\mu$ M Oligo-dT and 100  
500  $\mu$ M random hexamers suspended to 6.5  $\mu$ L dH<sub>2</sub>O was incubated at 65°C for five min followed by  
501 ice bath. Clontech SMART MMLV RT 5xFirst Strand buffer, 10 mM dNTP mix 100 mM  
502 DTT, and SMART MMLV RT was then added as per the manufacturer's protocol. Samples  
503 were then incubated at 42°C for 2 hrs followed by 85°C for five min to heat kill the RT.

504 Reverse transcribed cDNA was then carried forward for qPCR via a BioRad CFX-  
505 Connect Real-Time PCR thermal-cycler. 3  $\mu$ L of cDNA was used in conjunction with the  
506 BioRad iTaq Universal SYBR Green Supermix as per the manufacturer's protocol for 10  $\mu$ L  
507 reactions in a semi-hard qPCR 96-well plate. Each sample was plated in triplicate on the 96-well  
508 plate. Known dilutions of RSV<sup>WT</sup> plasmid were used for standard curve calculation. Using the  
509 CFX Maestro software, data was exported as an Excel spreadsheet.

510

511 *Data analysis*

512 Flow cytometry data was analyzed using FlowJo™ software (53). Values for  
513 fluorescence were exported to Excel spreadsheet. Images were analyzed using Fiji (ImageJ)  
514 (54). Western blot images were converted to 8-bit, Fiji's gel analysis tools used to calculate  
515 density, and values exported to an Excel spreadsheet. qPCR data was outputted into Excel  
516 spreadsheets. Excel spreadsheets were formatted for statistical analysis via R (55) and exported

517 to CSV format. RStudio was used to analyze data and create figures (56). UGene was used for  
518 plasmid cloning, sequence analysis, and multiple sequence alignment image generation (57).  
519 Final figures were prepared using Inkscape (58).

520

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526



527 **References**

- 528 1. Henderson BR, Percipalle P. 1997. Interactions between HIV Rev and nuclear import and  
529 export factors: the Rev nuclear localisation signal mediates specific binding to human  
530 importin-beta. *J Mol Biol* 274:693–707.
- 531 2. Truant R, Cullen BR. 1999. The arginine-rich domains present in human immunodeficiency  
532 virus type 1 Tat and Rev function as direct importin beta-dependent nuclear localization  
533 signals. *Mol Cell Biol* 19:1210–1217.
- 534 3. Neville M, Stutz F, Lee L, Davis LI, Rosbash M. 1997. The importin-beta family member  
535 Crm1p bridges the interaction between Rev and the nuclear pore complex during nuclear  
536 export. *Curr Biol* 7:767–775.
- 537 4. Wolff B, Sanglier JJ, Wang Y. 1997. Leptomycin B is an inhibitor of nuclear export:  
538 inhibition of nucleo-cytoplasmic translocation of the human immunodeficiency virus type 1  
539 (HIV-1) Rev protein and Rev-dependent mRNA. *Chem Biol* 4:139–147.
- 540 5. Bray M, Prasad S, Dubay JW, Hunter E, Jeang KT, Rekosh D, Hammarskjöld ML. 1994. A  
541 small element from the Mason-Pfizer monkey virus genome makes human  
542 immunodeficiency virus type 1 expression and replication Rev-independent. *Proc Natl*  
543 *Acad Sci U S A* 91:1256–1260.
- 544 6. Zolotukhin AS, Valentin A, Pavlakis GN, Felber BK. 1994. Continuous propagation of  
545 RRE(-) and Rev(-)RRE(-) human immunodeficiency virus type 1 molecular clones  
546 containing a cis-acting element of simian retrovirus type 1 in human peripheral blood  
547 lymphocytes. *J Virol* 68:7944–7952.

- 548 7. Ernst RK, Bray M, Rekosh D, Hammarskjöld ML. 1997. A structured retroviral RNA  
549 element that mediates nucleocytoplasmic export of intron-containing RNA. *Mol Cell Biol*  
550 17:135–144.
- 551 8. Sakuma T, Davila JI, Malcolm JA, Kocher J-PA, Tonne JM, Ikeda Y. 2014. Murine  
552 leukemia virus uses NXF1 for nuclear export of spliced and unspliced viral transcripts. *J*  
553 *Virol* 88:4069–4082.
- 554 9. Parent LJ. 2011. New insights into the nuclear localization of retroviral Gag proteins.  
555 *Nucleus* 2:92–97.
- 556 10. Kaddis Maldonado RJ, Parent LJ. 2016. Orchestrating the Selection and Packaging of  
557 Genomic RNA by Retroviruses: An Ensemble of Viral and Host Factors. *Viruses* 8.
- 558 11. Stake MS, Bann DV, Kaddis RJ, Parent LJ. 2013. Nuclear trafficking of retroviral RNAs  
559 and Gag proteins during late steps of replication. *Viruses* 5:2767–2795.
- 560 12. Scheifele LZ, Garbitt RA, Rhoads JD, Parent LJ. 2002. Nuclear entry and CRM1-dependent  
561 nuclear export of the Rous sarcoma virus Gag polyprotein. *Proc Natl Acad Sci U S A*  
562 99:3944–3949.
- 563 13. Scheifele LZ, Ryan EP, Parent LJ. 2005. Detailed mapping of the nuclear export signal in  
564 the Rous sarcoma virus Gag protein. *J Virol* 79:8732–8741.
- 565 14. Butterfield-Gerson KL, Scheifele LZ, Ryan EP, Hopper AK, Parent LJ. 2006. Importin-beta  
566 family members mediate alpharetrovirus gag nuclear entry via interactions with matrix and  
567 nucleocapsid. *J Virol* 80:1798–1806.

- 568 15. Lochmann TL, Bann DV, Ryan EP, Beyer AR, Mao A, Cochrane A, Parent LJ. 2013. NC-  
569 mediated nucleolar localization of retroviral gag proteins. *Virus Res* 171:304–318.
- 570 16. Rice BL, Stake MS, Parent LJ. 2020. TNPO3-Mediated Nuclear Entry of the Rous Sarcoma  
571 Virus Gag Protein Is Independent of the Cargo-Binding Domain. *Journal of Virology* 94.
- 572 17. Garbitt RA, Bone KR, Parent LJ. 2004. Insertion of a classical nuclear import signal into  
573 the matrix domain of the Rous sarcoma virus Gag protein interferes with virus replication. *J*  
574 *Virol* 78:13534–13542.
- 575 18. Garbitt-Hirst R, Kenney SP, Parent LJ. 2009. Genetic evidence for a connection between  
576 Rous sarcoma virus gag nuclear trafficking and genomic RNA packaging. *J Virol* 83:6790–  
577 6797.
- 578 19. Garbitt RA, Albert JA, Kessler MD, Parent LJ. 2001. trans-acting inhibition of genomic  
579 RNA dimerization by Rous sarcoma virus matrix mutants. *J Virol* 75:260–268.
- 580 20. Callahan EM, Wills JW. 2003. Link between genome packaging and rate of budding for  
581 Rous sarcoma virus. *J Virol* 77:9388–9398.
- 582 21. Gudleski N, Flanagan JM, Ryan EP, Bewley MC, Parent LJ. 2010. Directionality of  
583 nucleocytoplasmic transport of the retroviral gag protein depends on sequential binding of  
584 karyopherins and viral RNA. *Proc Natl Acad Sci U S A* 107:9358–9363.
- 585 22. Ogert RA, Lee LH, Beemon KL. 1996. Avian retroviral RNA element promotes unspliced  
586 RNA accumulation in the cytoplasm. *J Virol* 70:3834–3843.

- 587 23. Ogert RA, Beemon KL. 1998. Mutational analysis of the rous sarcoma virus DR  
588 posttranscriptional control element. *J Virol* 72:3407–3411.
- 589 24. LeBlanc JJ, Uddowla S, Abraham B, Clatterbuck S, Beemon KL. 2007. Tap and Dbp5, but  
590 not Gag, are involved in DR-mediated nuclear export of unspliced Rous sarcoma virus  
591 RNA. *Virology* 363:376–386.
- 592 25. Simpson SB, Zhang L, Craven RC, Stoltzfus CM. 1997. Rous sarcoma virus direct repeat  
593 cis elements exert effects at several points in the virus life cycle. *J Virol* 71:9150–9156.
- 594 26. Simpson SB, Guo W, Winistorfer SC, Craven RC, Stoltzfus CM. 1998. The upstream,  
595 direct repeat sequence of Prague A Rous sarcoma virus is deficient in mediating efficient  
596 Gag assembly and particle release. *Virology* 247:86–96.
- 597 27. Aschoff JM, Foster D, Coffin JM. 1999. Point mutations in the avian sarcoma/leukosis  
598 virus 3' untranslated region result in a packaging defect. *J Virol* 73:7421–7429.
- 599 28. Baluyot MF, Grosse SA, Lyddon TD, Janaka SK, Johnson MC. 2012. CRM1-dependent  
600 trafficking of retroviral Gag proteins revisited. *J Virol* 86:4696–4700.
- 601 29. Vlach J, Saad JS. 2015. Structural and molecular determinants of HIV-1 Gag binding to the  
602 plasma membrane. *Front Microbiol* 6:232.
- 603 30. Dick RA, Vogt VM. 2014. Membrane interaction of retroviral Gag proteins. *Front*  
604 *Microbiol* 5:187.

- 605 31. Nadaraia-Hoke S, Bann DV, Lochmann TL, Gudleski-O'Regan N, Parent LJ. 2013.  
606 Alterations in the MA and NC domains modulate phosphoinositide-dependent plasma  
607 membrane localization of the Rous sarcoma virus Gag protein. *J Virol* 87:3609–3615.
- 608 32. Doktorova M, Heberle FA, Kingston RL, Khelashvili G, Cuendet MA, Wen Y, Katsaras J,  
609 Feigenson GW, Vogt VM, Dick RA. 2017. Cholesterol Promotes Protein Binding by  
610 Affecting Membrane Electrostatics and Solvation Properties. *Biophys J* 113:2004–2015.
- 611 33. Chang LJ, Stoltzfus CM. 1985. Cloning and nucleotide sequences of cDNAs spanning the  
612 splice junctions of Rous sarcoma virus mRNAs. *J Virol* 53:969–972.
- 613 34. Mercredi PY, Bucca N, Loeliger B, Gaines CR, Mehta M, Bhargava P, Tedbury PR,  
614 Charlier L, Floquet N, Muriaux D, Favard C, Sanders CR, Freed EO, Marchant J, Summers  
615 MF. 2016. Structural and Molecular Determinants of Membrane Binding by the HIV-1  
616 Matrix Protein. *J Mol Biol* 428:1637–1655.
- 617 35. Göttlinger HG, Sodroski JG, Haseltine WA. 1989. Role of capsid precursor processing and  
618 myristoylation in morphogenesis and infectivity of human immunodeficiency virus type 1.  
619 *Proc Natl Acad Sci U S A* 86:5781–5785.
- 620 36. Hill CP, Worthylake D, Bancroft DP, Christensen AM, Sundquist WI. 1996. Crystal  
621 structures of the trimeric human immunodeficiency virus type 1 matrix protein:  
622 implications for membrane association and assembly. *Proc Natl Acad Sci USA* 93:3099–  
623 3104.

- 624 37. Cui N, Su S, Chen Z, Zhao X, Cui Z. 2014. Genomic sequence analysis and biological  
625 characteristics of a rescued clone of avian leukosis virus strain JS11C1, isolated from  
626 indigenous chickens. *J Gen Virol* 95:2512–2522.
- 627 38. Kempf N, Postupalenko V, Bora S, Didier P, Arntz Y, de Rocquigny H, Mély Y. 2015. The  
628 HIV-1 nucleocapsid protein recruits negatively charged lipids to ensure its optimal binding  
629 to lipid membranes. *J Virol* 89:1756–1767.
- 630 39. Dick RA, Datta SAK, Nanda H, Fang X, Wen Y, Barros M, Wang Y-X, Rein A, Vogt VM.  
631 2015. Hydrodynamic and Membrane Binding Properties of Purified Rous Sarcoma Virus  
632 Gag Protein. *J Virol* 89:10371–10382.
- 633 40. Dick RA, Barros M, Jin D, Lösche M, Vogt VM. 2015. Membrane Binding of the Rous  
634 Sarcoma Virus Gag Protein Is Cooperative and Dependent on the Spacer Peptide Assembly  
635 Domain. *J Virol* 90:2473–2485.
- 636 41. Jin J, Sturgeon T, Weisz OA, Mothes W, Montelaro RC. 2009. HIV-1 matrix dependent  
637 membrane targeting is regulated by Gag mRNA trafficking. *PLoS One* 4:e6551.
- 638 42. Sun M, Grigsby IF, Gorelick RJ, Mansky LM, Musier-Forsyth K. 2014. Retrovirus-specific  
639 differences in matrix and nucleocapsid protein-nucleic acid interactions: implications for  
640 genomic RNA packaging. *J Virol* 88:1271–1280.
- 641 43. Lee E, Alidina A, May C, Linial ML. 2003. Importance of basic residues in binding of rous  
642 sarcoma virus nucleocapsid to the RNA packaging signal. *J Virol* 77:2010–2020.

- 643 44. Lee E-G, Linial ML. 2004. Basic residues of the retroviral nucleocapsid play different roles  
644 in gag-gag and Gag-Psi RNA interactions. *J Virol* 78:8486–8495.
- 645 45. Engelman A, Cherepanov P. 2012. The structural biology of HIV-1: mechanistic and  
646 therapeutic insights. *Nat Rev Microbiol* 10:279–290.
- 647 46. Freed EO. 2015. HIV-1 assembly, release and maturation. *Nat Rev Microbiol* 13:484–496.
- 648 47. El Meshri SE, Dujardin D, Godet J, Richert L, Boudier C, Darlix JL, Didier P, Mély Y, de  
649 Rocquigny H. 2015. Role of the nucleocapsid domain in HIV-1 Gag oligomerization and  
650 trafficking to the plasma membrane: a fluorescence lifetime imaging microscopy  
651 investigation. *J Mol Biol* 427:1480–1494.
- 652 48. Stewart-Maynard KM, Cruceanu M, Wang F, Vo M-N, Gorelick RJ, Williams MC,  
653 Rouzina I, Musier-Forsyth K. 2008. Retroviral nucleocapsid proteins display nonequivalent  
654 levels of nucleic acid chaperone activity. *J Virol* 82:10129–10142.
- 655 49. Lyonnais S, Gorelick RJ, Heniche-Boukhalfa F, Bouaziz S, Parissi V, Mouscadet J-F,  
656 Restle T, Gatell JM, Le Cam E, Mirambeau G. 2013. A protein ballet around the viral  
657 genome orchestrated by HIV-1 reverse transcriptase leads to an architectural switch: from  
658 nucleocapsid-condensed RNA to Vpr-bridged DNA. *Virus Res* 171:287–303.
- 659 50. Jorgenson RL, Vogt VM, Johnson MC. 2009. Foreign glycoproteins can be actively  
660 recruited to virus assembly sites during pseudotyping. *J Virol* 83:4060–4067.

- 661 51. Chang L-J, Urlacher V, Iwakuma T, Cui Y, Zucali J. 1999. Efficacy and safety analyses of  
662 a recombinant human immunodeficiency virus type 1 derived vector system. *Gene Ther*  
663 6:715–728.
- 664 52. 2006. RIPA buffer. *Cold Spring Harb Protoc* 2006:pdb.rec10035.
- 665 53. 2021. FlowJo™ software for Mac. Becton, Dickinson and Company, Ashland, OR.
- 666 54. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S,  
667 Rueden C, Saalfeld S, Schmid B, Tinevez J-Y, White DJ, Hartenstein V, Eliceiri K,  
668 Tomancak P, Cardona A. 2012. Fiji: an open-source platform for biological-image analysis.  
669 *Nat Methods* 9:676–682.
- 670 55. R Core Team. 2021. R: A Language and Environment for Statistical Computing. R  
671 Foundation for Statistical Computing, Vienna, Austria.
- 672 56. RStudio Team. 2015. RStudio: Integrated Development Environment for R. RStudio, Inc.,  
673 Boston, MA.
- 674 57. Okonechnikov K, Golosova O, Fursov M, UGENE team. 2012. Unipro UGENE: a unified  
675 bioinformatics toolkit. *Bioinformatics* 28:1166–1167.
- 676 58. 2021. Inkscape.
- 677



678 **Figure Legends**

679

680 **Figure 1. Subcellular expression of various Gag-GFP fusion proteins.**

681 (A) Schematic of Gag-GFP fusion proteins tested. RSV<sup>WT</sup>, RSV<sup>L219A</sup> (lacks a functional NES),  
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683 parallel. H/RSV-Gag<sup>WT</sup> was further probed with the G2A mutation (blocks myristoylation thus  
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692 post transfection and (C) imaged after LMB (10 ng/mL) treatment for 1 hr.

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694 **Figure 2. RSV-Gag<sup>K36E/M</sup> mutants are defective in nuclear cycling.**

695 (A) Multiple sequence alignment of RSV-NC between the WT consensus and strain JS11C1.  
696 There is a K36E polymorphism in the NC-NLS. Since Glu is the opposite charge from Lys, both  
697 Glu and Met mutations were tested for nuclear import activity. Mutations were made in the WT  
698 backbone (panel A). (B) Representative images of steady state expression of RSV-Gag<sup>WT</sup> and  
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701 previous panel after LMB (10 ng/mL) treatment for 1 hr.

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703 **Figure 3. Gag NLS mutants are infectious in single-cycle virus.**

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705 Crimson fluorescence gene is located outside of the viral genome (indicated by the LTRs),  
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713 Since RSV<sup>ΔEnv/K36M</sup> displayed near RSV<sup>ΔEnv</sup> levels of spread compared to RSV<sup>ΔEnv/K36E</sup> in Fig 3,  
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716 Replication competent virus spread through cell culture as measured by percent GFP (+) cells.  
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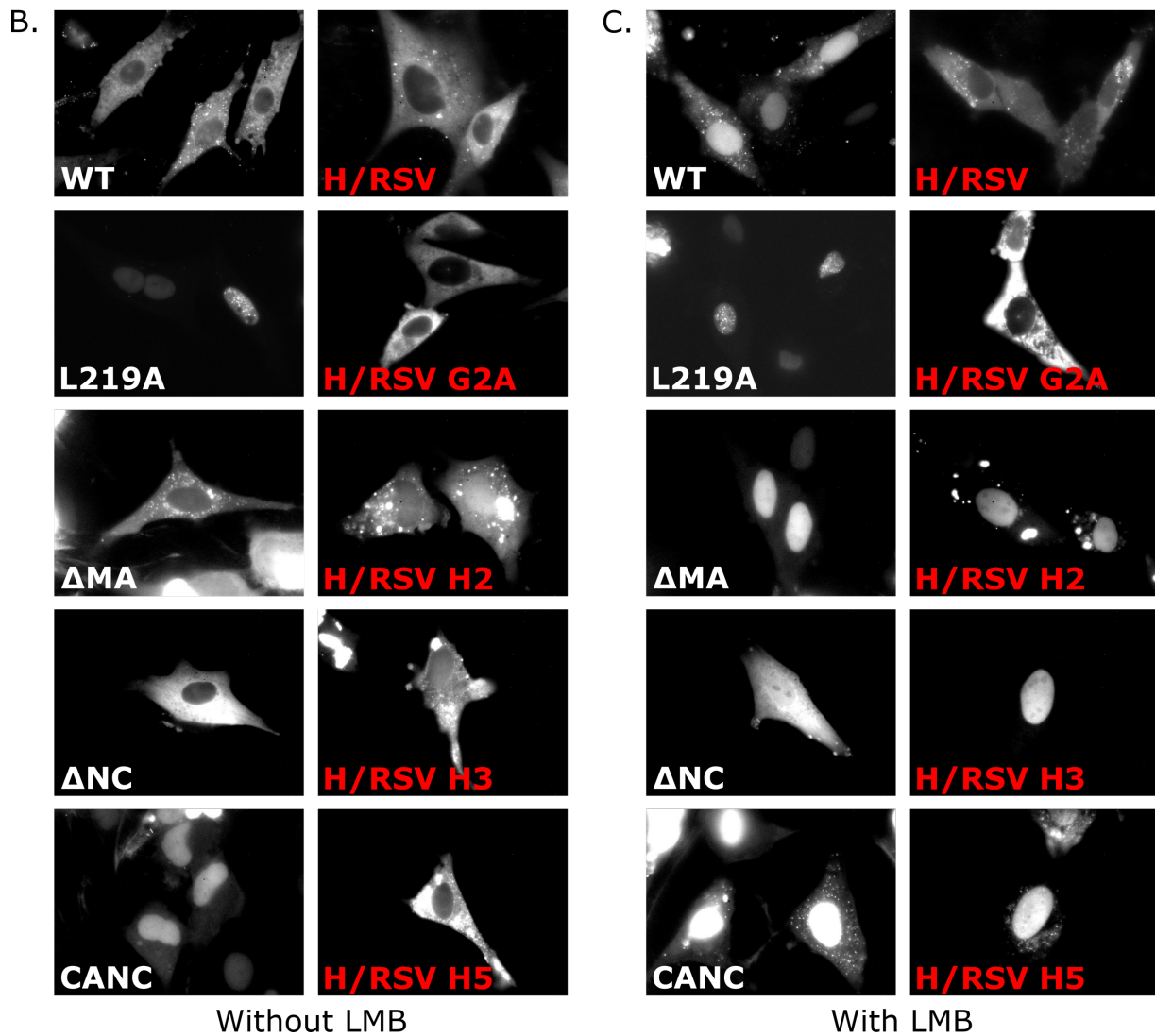
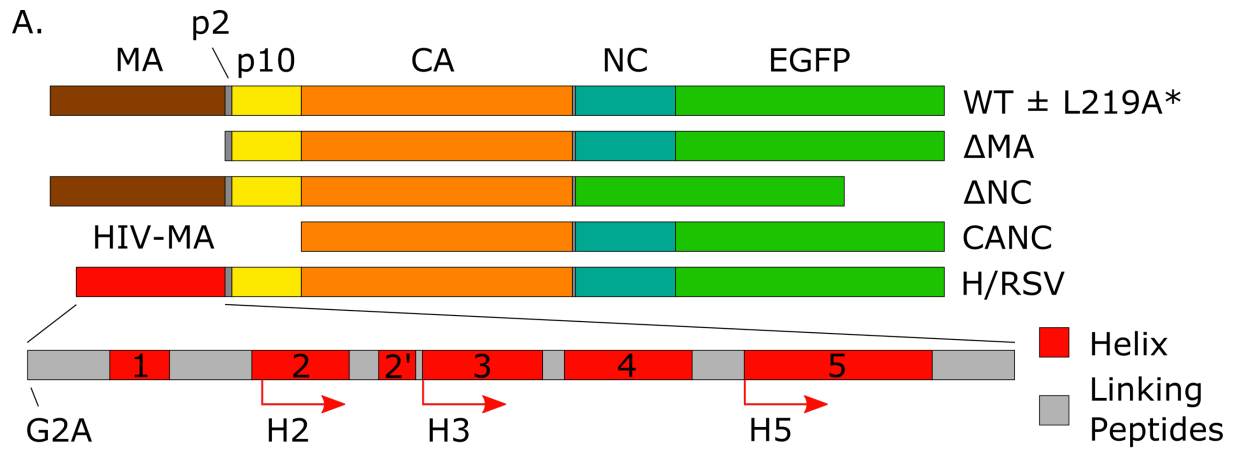
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733 **Figure 6. Genomic RNA incorporation in viral particles does not differ between WT and**  
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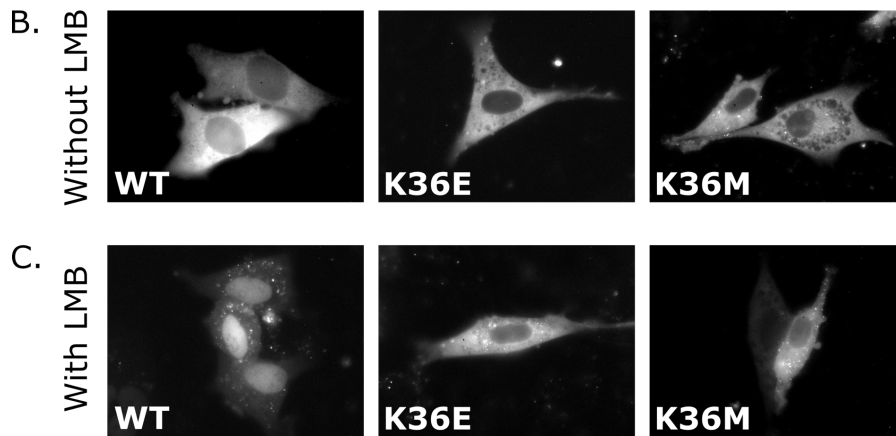
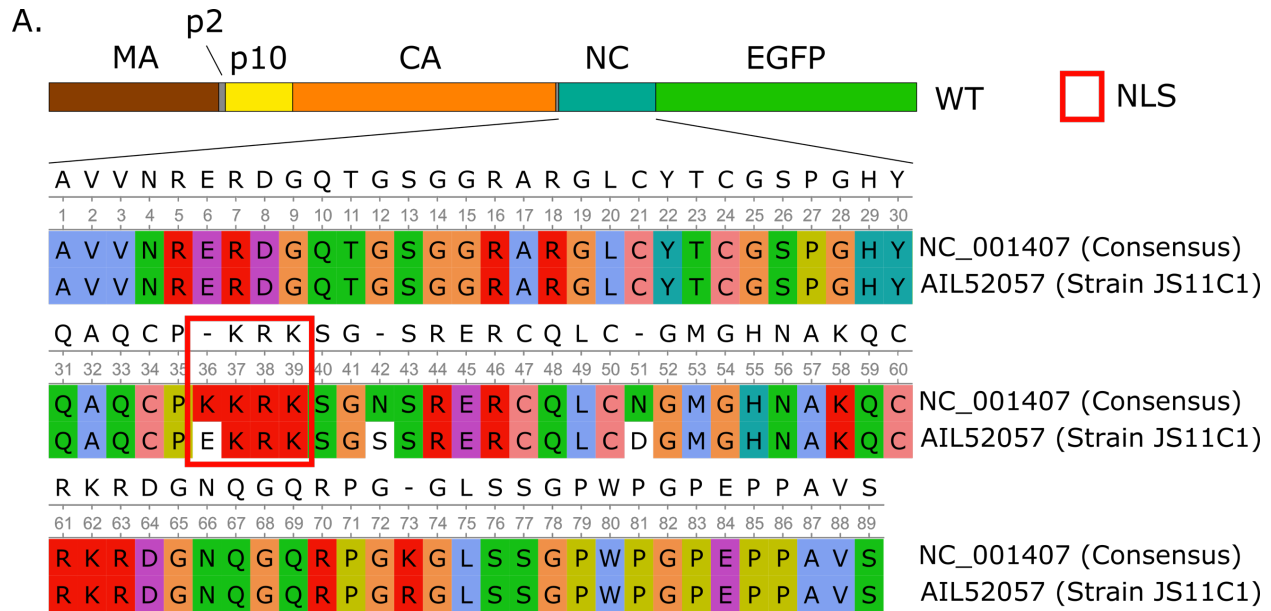
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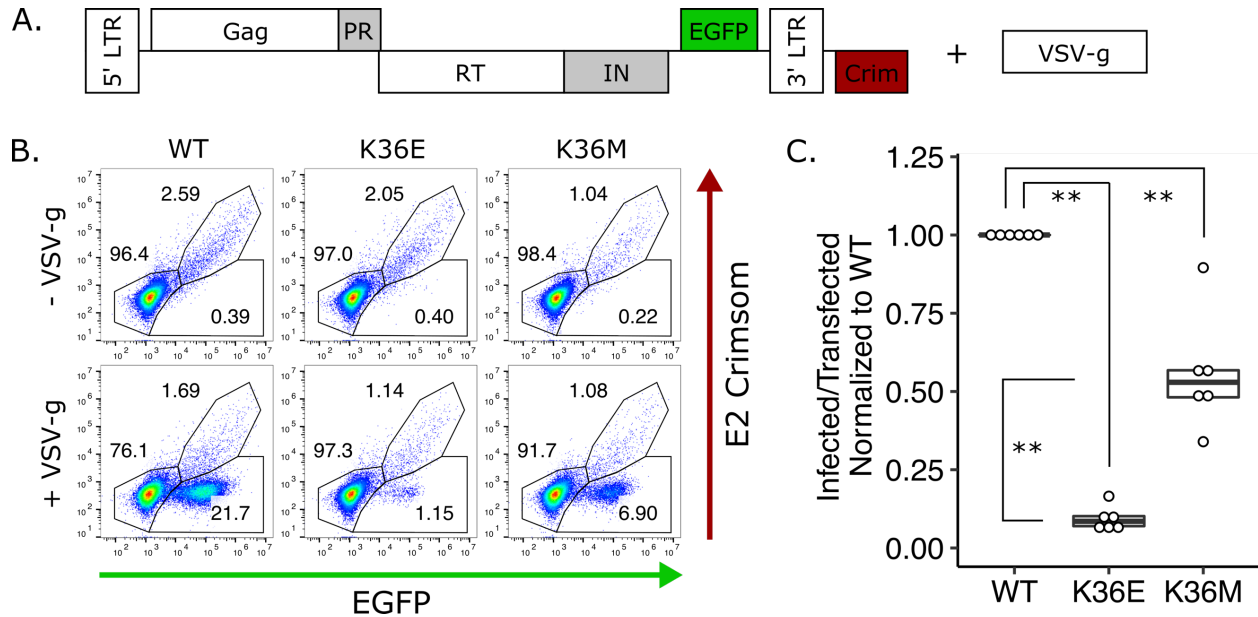
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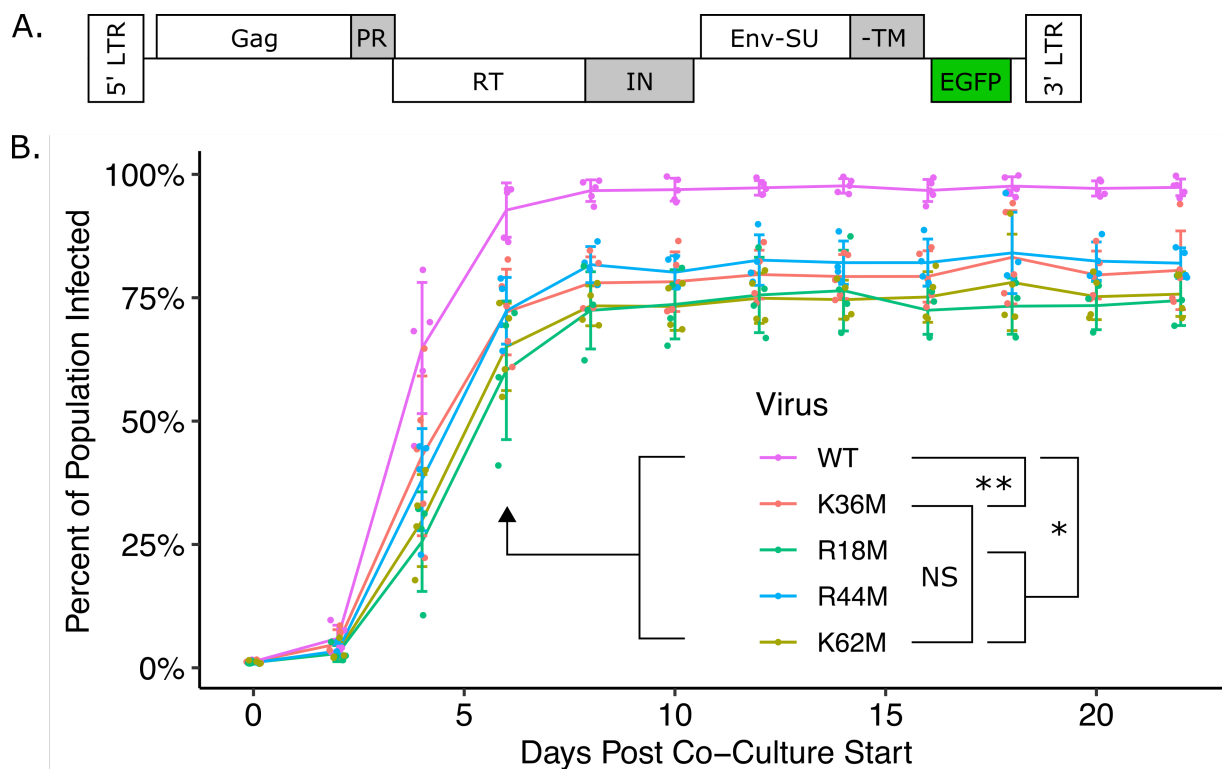
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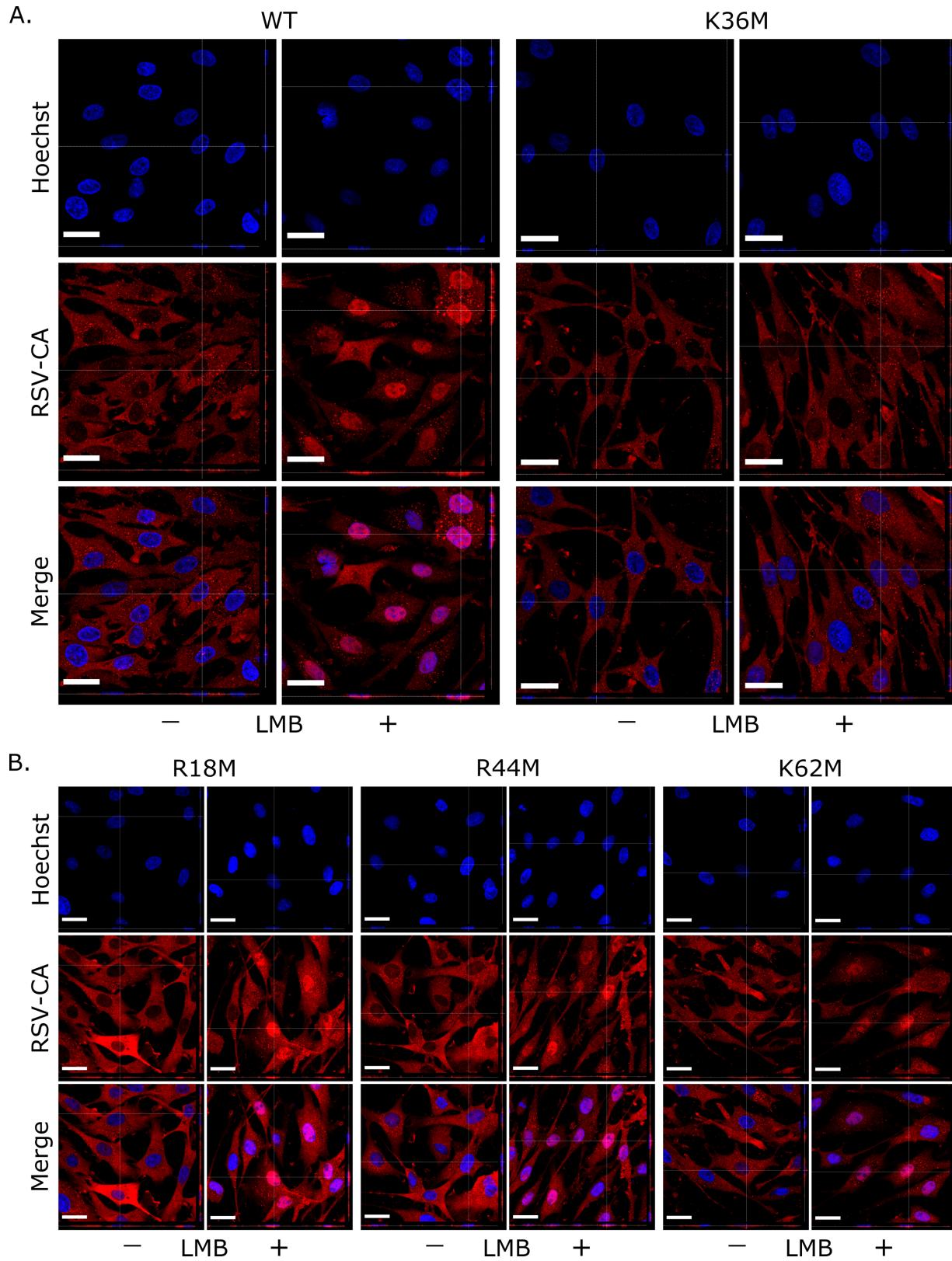
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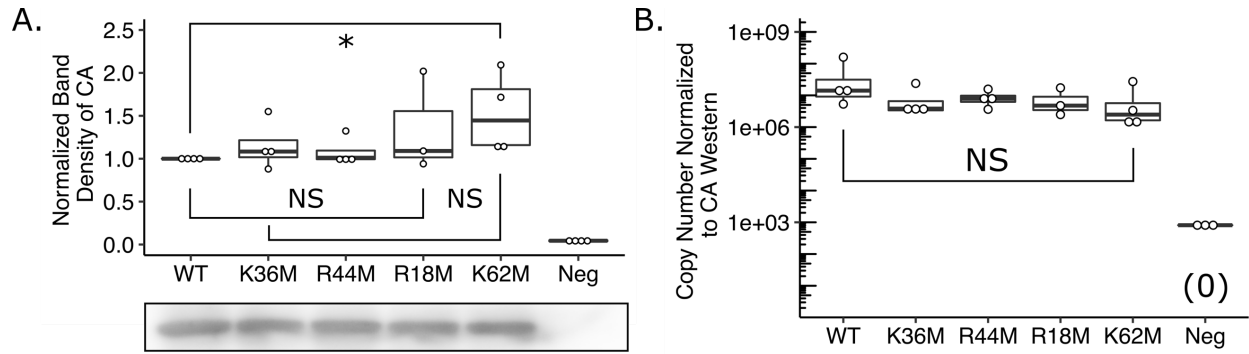
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