Title:

HIV-1 initiates genomic RNA packaging in a unique subset of host RNA granules

Motoko Tanaka, Brook C. Barajas, Bridget A. Robinson, Daryl Phuong, Kasana Chutiraka, Jonathan C. Reed, and Jaisri R. Lingappa

Department of Global Health, University of Washington, Seattle, WA 98109

Short Title:

HIV-1 gRNA packaging occurs in unique host RNA granules

Abbreviations used:

αABCE1, antibodies directed against ABCE1

αDDX6, antibodies directed against DDX6

αGag, antibodies directed against Gag

αGFP, antibodies directed against GFP

coIP, coimmunoprecipitation

gRNA, HIV-1 genomic RNA

IEM, immunoelectron microscopy

IP, immunoprecipitation

LZ, leucine zipper

NC, nucleocapsid

O-FISH, oligo fluorescent in situ hybridization

O-FISH PLA, oligo fluorescent in situ hybridization coupled with the proximity ligation assay

PLA, proximity ligation assay

PM, plasma membrane

PuroHS, treatment with puromycin plus high salt

RNPC, ribonucleoprotein

RT-qPCR

SRP, signal recognition particle

VLPs, virus-like particles

WB, Western blot

WT, wild type

Abstract

How HIV-1 genomic RNA (gRNA) is packaged into assembling virus remains unclear. Here, we use biochemical and *in situ* approaches to identify the complex in which the capsid protein Gag first associates with gRNA, termed the packaging initiation complex. First, we show that in the absence of assembling Gag, non-nuclear non-translating gRNA is nearly absent from the soluble fraction of provirus-expressing cells, and is found instead primarily in complexes ≥30S. When we express a Gag mutant known to be arrested at packaging initiation, we find only one complex containing Gag and gRNA; thus, this complex corresponds to the packaging initiation complex. This ~80S complex also contains two cellular facilitators of assembly, ABCE1 and the RNA granule protein DDX6, and therefore corresponds to a co-opted host RNA granule and a previously described capsid assembly intermediate. Additionally, we find this granule-derived packaging initiation complex in HIV-1-infected H9 T cells, and demonstrate that wild-type Gag forms both the packaging initiation complex and a larger granule-derived complex corresponding to a late packaging/assembly intermediate. We also demonstrate that packaging initiation complexes are far more numerous than P bodies *in situ*. Finally, we show that Gag enters the ~80S granule to form the packaging initiation complex via a two-step mechanism. In a step that is independent of a gRNA-binding domain, Gag enters a broad class of RNA granules, most of which lack gRNA. In a second step that is dependent on the gRNA-binding nucleocapsid domain of Gag or a heterologous gRNA-binding domain, Gag enters a gRNA-containing subset of these granules. Thus, we conclude that packaging in cells does not result from random encounters between Gag and gRNA; instead our data support a fundamentally different model in which Gag is directed to gRNA within a unique host RNA granule to initiate this critical event in HIV-1 replication.

Nontechnical Summary

To form infectious virus, the HIV-1 capsid protein Gag must associate with and package the viral genomic RNA (gRNA) during the virus assembly process. HIV-1 Gag first associates with gRNA in the cytoplasm, forming a complex termed the packaging initiation complex; this complex subsequently targets to the plasma membrane where Gag completes the assembly and packaging process before releasing the virus from the cell. Although the packaging initiation complex is critical for infectious virus formation, its identity and composition, and the mechanism by which it is formed, remain unknown. Here we identify the packaging initiation complex, and demonstrate that it corresponds to a host RNA granule that is co-opted by the virus. RNA granules are diverse complexes utilized by host cells for all aspects of RNA storage and metabolism besides translation. Our study also defines the mechanism by which HIV-1 Gag enters this host RNA granule to form the packaging initiation complex, and reveal that it involves two steps that depend on different regions of Gag. Our finding that Gag co-opts a poorly studied host complex to first associate with gRNA during packaging provides a new paradigm for understanding this critical event in the viral life cycle.

105 **Introduction**

106 For released HIV-1 particles to be infectious, two copies of full-length genomic RNA (gRNA)
107 must be packaged during assembly of the immature HIV-1 capsid. Capsid assembly involves
108 oligomerization of Gag in the cytoplasm followed by targeting of Gag via its N-terminal myristate to the
109 plasma membrane (PM), where ~3000 Gag proteins multimerize to form each immature capsid.
110 Packaging of gRNA is initiated when the structural protein Gag first associates with gRNA during
111 assembly, and requires the nucleocapsid domain (NC) of Gag as well as specific encapsidation signals in
112 gRNA (reviewed in [1]). The gRNA-containing immature capsids subsequently undergo budding,
113 release, and maturation (reviewed in [2]). In addition to being used for packaging, HIV-1 gRNA in the
114 cytoplasm is also used for translation of Gag and GagPol (reviewed in [1]). Translation and packaging
115 are thought to be mutually exclusive, with the shift between these processes likely controlled by a gRNA
116 conformational switch (reviewed in [3,4]). Thus, gRNAs that are packaged are likely to be non-
117 translating gRNAs with unique structural features [5]. In the absence of gRNA, capsid assembly and
118 release still occur but the resulting virus is non-infectious [6].

119 The process by which HIV-1 Gag finds gRNA at the start of packaging is of great interest.
120 Biochemical and imaging studies have established that a Gag dimer or oligomer first associates with
121 gRNA in the cytoplasm, forming a complex termed the packaging initiation complex [7]. Subsequently,
122 the packaging initiation complex targets to the PM where Gag multimerizes to form the immature capsid
123 [8]. Live imaging studies have defined the diffusion characteristics of Gag and gRNA in cells [9,10]; but,
124 notably, the exact components in these diffusing complexes have not been identified. Thus, although
125 formation of the packaging initiation complex is critical for production of infectious virus, the
126 composition of the packaging initiation complex and the mechanism by which Gag and gRNA first
127 associate to form this complex are not known.

128 Here we used our understanding of HIV-1 capsid assembly to determine how the packaging
129 initiation complex is formed. Our previous studies showed that assembling HIV-1 Gag co-opts RNA
130 granules [11], which are host ribonucleoprotein complexes (RNPCs) that contain non-translating mRNAs.

131 While all translating cellular mRNAs are in monosomes or polysomes, non-translating cellular mRNAs
132 reside in RNA granules (reviewed in [12]). Numerous types of RNA granules exist; these granules are
133 distinguished by sizes and marker proteins, and function in silencing, storage, degradation, stress, and
134 other events in RNA metabolism. Some RNA granules, such as P bodies and stress granules, are easily
135 visible by light microscopy, but others are smaller and poorly understood. We found that after co-opting
136 host RNA granules, Gag remains in these granules during immature capsid assembly, forming a
137 sequential pathway of assembly intermediates named by their sedimentation values of ~80S, ~150S, and
138 ~500S (reviewed in [13]). Both the original co-opted host RNA granule and the subsequently formed
139 assembly intermediates contain multiple RNA granule proteins, including the DEAD-box RNA helicase
140 DDX6 [11].

141 These previous studies led us to what we term the RNA granule model of packaging. In this
142 model, we propose that, like non-translating cellular mRNA, non-translating gRNAs are sequestered
143 within RNA granules; thus, Gag must enter gRNA-containing host RNA granules to initiate gRNA
144 packaging. Localizing packaging to RNA granules could be advantageous to the virus in a number of
145 ways: it would sequester gRNA from the host innate immune system, concentrate Gag at the site where
146 gRNA is located, and place packaging and assembly in proximity with host enzymes that could facilitate
147 those events. In keeping with the latter possibility, two of the host proteins present in both the assembly
148 intermediates and the host RNA granules from which they are derived are cellular facilitators of HIV-1
149 capsid assembly – the ATP-binding cassette protein E1 (ABCE1) and DDX6 [11,14].

150 Here we identified the packaging initiation complex and tested the RNA granule model of HIV-1
151 packaging. First we showed that all non-translating HIV-1 gRNAs are in large complexes, even in the
152 absence of assembling Gag. Using a Gag mutant that is arrested after packaging initiation, we
153 demonstrated that only one complex contains Gag associated with gRNA, and therefore fits the definition
154 of the packaging initiation complex. This complex is an ~80S RNA granule that also contains the cellular
155 facilitators of assembly, ABCE1 and DDX6. *In situ* studies confirmed these findings and revealed that
156 the packaging initiation complexes are smaller and more numerous than P bodies. Additionally, we

157 examined the mechanism by which the packaging initiation complex is formed, and found that Gag uses
158 both a gRNA-binding-independent and a gRNA-binding-dependent step to localize to gRNA-containing
159 RNA granules. Finally, we confirmed the physiological relevance of the RNA-granule derived packaging
160 intermediates by identifying them in a chronically infected human T cell line. Together, our data argue
161 that packaging of the HIV-1 genome is initiated only after Gag localizes to a unique and poorly
162 understood subclass of host RNA granules that contains non-translating gRNA.
163

164 **Results**

165 **Confirmation of packaging phenotypes for proviruses expressing WT Gag and Gag mutants**

166 To study gRNA packaging, we used a variety of gRNA expression systems (Fig 1A) that
167 produce either WT Gag or Gag mutants with known phenotypes (Fig 1B). WT Gag forms the packaging
168 initiation complex and completes assembly to produce virus-like particles (VLPs) that contain gRNA.
169 Two assembly-defective Gag mutants, MACA Gag and G2A Gag, were studied because they arrest Gag
170 assembly before or after packaging initiation complex formation, respectively. The assembly-
171 incompetent MACA Gag fails to form the packaging initiation complex because it lacks the gRNA-
172 binding NC domain (reviewed in [13,15,16]). In contrast, the assembly-defective G2A Gag forms the
173 cytoplasmic packaging initiation complex [7] but is arrested in the cytoplasm due to a point mutation that
174 prevents the myristoylation required for PM targeting [17-19]. We also analyzed the assembly-competent
175 but gRNA-interaction-deficient HIV-1 GagZip chimera. In place of the gRNA-binding NC domain,
176 GagZip contains a dimerizing leucine zipper (LZ), which supports assembly but not RNA association [20-
177 24]; thus GagZip produces VLPs that lack gRNA, and would not be expected to form the packaging
178 initiation complex. GagZip is of interest since a packaging model should be able to explain how GagZip
179 successfully assembles capsids but fails to incorporate gRNA.

180 We confirmed the VLP phenotypes described above following transfection of WT and mutant
181 proviruses (Fig 1A, Set I) into COS-1 (Fig 1C) by analyzing cell lysates and VLPs for Gag and gRNA
182 copies (Fig 1C, top). Additionally, the predicted packaging initiation complex phenotypes of these four

183 Gag constructs (Fig 1B) were confirmed by quantifying gRNA copies associated with intracellular Gag,
184 using immunoprecipitation (IP) from cell lysates with antibody directed against Gag (αGag), followed by
185 RTqPCR of IP eluates (Fig 1C, bottom).
186
187 **Non-translating gRNAs are in diverse large complexes even in the absence of assembling Gag**
188 To date, the spectrum of gRNA-containing complexes present in the cytoplasm has not been
189 defined. Our model predicts that gRNA behaves like mRNA; thus, even in the absence of Gag we would
190 expect to find that all HIV-1 gRNA is either in translating complexes or in diverse non-translating
191 RNPCs. Moreover, because gRNA, like mRNA, should associate with ribonucleoproteins to form
192 relatively large complexes, we would not expect to find gRNA in the soluble fraction of cell lysates,
193 which contains monomers, dimers, and small oligomers. To test these predictions, we transfected cells
194 with MACA provirus (Fig 2A), which expresses an otherwise full-length gRNA encoding a truncated
195 assembly-incompetent MACA Gag protein that is arrested as an ~10S complex [25]. These cells were
196 harvested under conditions that removed nuclei, kept RNPCs intact, and solubilized any membranes
197 associated with proteins. Lysates were analyzed by velocity sedimentation followed by RT-qPCR to
198 determine the approximate size (S value) of all non-nuclear RNPCs containing gRNA (or other RNAs).
199 This analysis was performed with both translating and non-translating complexes intact, and also
200 following disruption of translating ribosomes with *puro*mycin and *h*igh *s*alt *t*reatment (PuroHS). PuroHS
201 releases nascent chains from ribosomes, dissociates ribosomal subunits, and releases mRNA [26,27],
202 which then likely shifts into non-translating RNPCs [28]. Thus, PuroHS treatment should allow analysis
203 of only non-translating gRNAs, the population likely to undergo packaging.
204 We first determined the efficacy of ribosome disruption by PuroHS. To do this, we analyzed
205 MACA gradient fractions for 28S rRNA, a marker for the 60S large ribosomal subunit (Fig 2B),
206 following PuroHS treatment or in the absence of PuroHS treatment. As expected, in the absence of
207 PuroHS treatment (Fig 2B, open green circles), 28S rRNA migrated almost entirely in the position of
208 monosomes (~80S) and polysomes (>150S). Following PuroHS treatment (Fig 2B, solid green squares),

209 28S rRNA migrated almost entirely in an ~60S peak representing the dissociated large ribosomal subunit,
210 with almost no 28S rRNA remaining in the polysome region (>150S). The near complete absence of 28S
211 rRNA in the polysome region after PuroHS treatment indicated highly effective ribosome disassembly.
212 From these data we concluded that PuroHS disassembles most 28S-containing monosomes and
213 polysomes into large and small ribosomal subunits. As a negative control, the same fractions were
214 examined for 7SL RNA, a marker for signal recognition particle (SRP), which is a ribosome-independent
215 ~11S RNPC [29]. As expected, PuroHS treatment did not affect the migration of 7SL (Fig 2B, compare
216 open orange circles vs. solid orange squares). Thus, RNPCs of different sizes (monosomes, polysomes,
217 and SRP) remain intact following cell lysis and velocity sedimentation, and PuroHS treatment disrupted
218 translating RNPCs, but not ribosome-independent RNPCs.

219 Analysis of HIV-1 gRNA in these same MACA fractions revealed that, with or without PuroHS
220 treatment, almost no HIV-1 gRNA was in the soluble fraction (≤ 20S) and very little was in small
221 complexes of < 30S (Fig 2C, compare open blue circles to solid blue squares). Thus, at steady state in the
222 absence of assembling Gag, most gRNA is in complexes ≥ 30S, consisting of translating complexes and
223 non-translating complexes. This contrasted with MACA protein, which was found almost entirely in the
224 soluble fraction (≤ 20S; Fig 2C, grey triangles). Following PuroHS treatment, > 95% of gRNA was in
225 diverse RNPCs ≥ 30S that formed a broad peak centered at ~60S. Comparison of gRNA data obtained
226 with and without PuroHS treatment suggests that approximately 30% of total gRNA was in translating
227 polysomes, defined as complexes ≥ 150S that are lost upon PuroHS. Moreover, PuroHS treatment caused
228 HIV-1 gRNA that was in polysomes (≥150S) to shift into non-translating RNPCs of ~30-150S, some of
229 which are present even in the absence of PuroHS treatment (Fig. 2C). When the same gradient fractions
230 were analyzed for HIV-1 subgenomic Tat mRNA and a cellular mRNA (GAPDH), a pattern similar to
231 that of HIV-1 gRNA was observed, with or without PuroHS treatment (compare Fig 2D, E to 2C),
232 although perhaps with GAPDH mRNA forming more ≥150S complexes after puromycin treatment,
233 which could correspond to non-translating RNA granules or residual polysomes not disrupted by
234 puromycin. Thus, the distribution of translating and non-translating gRNA-containing complexes

235 resembles the distribution of cellular mRNAs and subgenomic viral mRNAs. Because packaging likely
236 involves non-translating gRNA, as explained above, all subsequent lysate experiments utilized PuroHS to
237 allow analysis of almost exclusively non-translating gRNA.
238
239 **G2A Gag enters gRNA-containing granules to form the ~80S packaging initiation complex**
240 Having shown that non-translating gRNA is primarily in RNPCs ≥ 30S, and does not comigrate
241 with soluble packaging-incompetent MACA Gag, we next asked whether a packaging-initiation-
242 competent Gag enters one of the ≥ 30S RNPCs to form the packaging initiation complex. Because the
243 packaging initiation complex is the complex in which Gag and gRNA first associate, we would expect the
244 packaging initiation complex to have two key features – first, gRNA and Gag should comigrate in the
245 fractions that contain the packaging initiation complex, and secondly, gRNA and Gag in these fractions
246 should be in association with each other, through direct or indirect interactions, as indicated by
247 coimmunoprecipitation (coIP). In addition, if the packaging initiation complex is a co-opted RNA
248 granule, we would expect gRNA to associate with RNA granule proteins in these fractions.
249 To help identify the packaging initiation complex, we utilized a provirus that expresses G2A Gag.
250 G2A Gag forms the cytoplasmic complex that has been defined as the packaging initiation complex [7],
251 and is likely arrested in the form of the packaging initiation complex due to its inability to target to
252 membranes. To demonstrate comigration of gRNA with packaging-initiation-competent G2A Gag, cells
253 expressing MACA vs. G2A proviruses (Fig 1A, Set I) to similar steady state levels (Fig 3A), were
254 analyzed by velocity sedimentation followed by quantitation of gRNA in gradient fractions (Fig 3B).
255 Cells expressing either the MACA or G2A provirus displayed the same distribution of total non-nuclear
256 non-translating gRNA, with gRNA mainly in ~30-80S RNPCs in both cases (Fig 3B, graph). In contrast,
257 the distribution of the MACA vs. G2A Gag protein across the gradient differed dramatically (Fig 3B, WB
258 below graph), as observed previously [25,30]. While MACA protein was almost entirely in the soluble
259 fraction (Fig 3B, MACA WB, fractions 1 - 3), G2A Gag protein was present in both the ≤ 20S soluble
260 fraction and in ~40-80S complexes (Fig 3B, G2A WB, fractions 8 – 12). Thus, a population of

261 packaging-initiation-competent G2A Gag protein comigrated with gRNA in ~40-80S RNPCs, in contrast
262 to the MACA protein, which is not competent for packaging initiation.

263 To determine whether gRNA in the ~40-80S RNPCs is associated with comigrating G2A Gag,
264 gradient fractions in Fig 3B were subjected to αGag IP, followed by quantitation of gRNA in IP eluates
265 (Fig 3C). Note that all IPs in this study were performed under native conditions, and are thus expected to
266 pull down the protein targeted by the antibody as well as other components stably associated with the
267 target protein through direct or indirect interactions. Even though αGag coimmunoprecipitated MACA
268 effectively (Fig 1C), no gRNA was associated with MACA by αGag IP in any fraction, as expected given
269 that MACA does not comigrate with gRNA (Fig 3B, compare graph to blot). Similarly, gRNA was not
270 associated with soluble G2A Gag protein (in the ≤ 20S region) by coIP, as expected given the lack of
271 gRNA in the soluble fractions. Thus, the packaging initiation complex was not found in soluble fractions.
272 In contrast, G2A Gag protein was strongly associated with gRNA by αGag coIP in a broad RNPC that
273 peaked at ~80S, heretofore referred to as the ~80S complex (Fig 3C, graph). From these data, we
274 conclude only one RNPC - the ~80S complex - fits the definition of the packaging initiation complex in
275 that it contains G2A Gag associated with gRNA.

276 Next we asked whether this ~80S RNPC is an RNA granule, by determining if gRNA in this
277 complex is associated with the RNA granule protein, DDX6. DDX6 is a marker for P bodies [31], but is
278 also found in ~80S and ~500S RNA granules that are co-opted by Gag during assembly and contain the
279 host enzyme ABCE1 [11]. In cells expressing G2A provirus (Fig 3D), gRNA was again observed
280 primarily in the ~40-80S region of gradients (Fig 3E). Moreover, antibody to DDX6 (αDDX6)
281 coimmunoprecipitated gRNA from a complex that peaks in the ~80S region (Fig 3F). These findings are
282 consistent with our previous observation that DDX6 is associated with Gag in the ~80S assembly
283 intermediate by coIP [11]. Taken together, these data suggest that to form the packaging initiation
284 complex, packaging-competent G2A Gag must enter an ~80S DDX6-containing RNA granule that
285 contains non-translating gRNA. Notably, gRNA is absent from the <20S fraction of the cell lysate, with
286 or without PuroHS treatment (Fig 2C), and in the presence or absence of packaging-initiation-competent

287 Gag (Fig 3B); thus we could find no evidence of a small complex containing only gRNA and a dimer of
288 Gag.
289
290 **WT Gag forms the ~80S packaging initiation complex and ~500S late packaging intermediate,**
291 **which contain RNA granule proteins**
292 Previously, we used pulse-chase and mutational approaches to demonstrate that WT Gag forms
293 an ~80S early capsid assembly intermediate, a ~500S late assembly intermediate, and a ~750S complex
294 that corresponds to a completed immature capsid [25,32-34]. Additionally, we had used coIPs to
295 demonstrate that the ~80S and ~500S assembly intermediates contain the assembly facilitators ABCE1
296 and DDX6 in association with Gag [11,14]. If the ~80S assembly intermediate is also the earliest
297 packaging intermediate, as suggested by the data above (Fig 3), then we would expect the ~500S
298 assembly intermediate, which is only found at the PM [25], to be a late packaging intermediate that
299 contains gRNA in association with targeting-competent Gag. Thus, WT Gag should be associated with
300 gRNA in both the ~80S packaging initiation complex/early assembly intermediate and the ~500S late
301 assembly/packaging intermediate, as well as in any intracellular ~750S completed immature capsids that
302 accumulate prior to virus budding and release. To test these predictions, we needed a method for
303 immunoprecipitating multimerized Gag. Since diverse Gag antibodies fail to IP multimerized Gag
304 because of epitope masking [35], we utilized αGFP IP of GFP-tagged, codon-optimized Gag (GagGFP)
305 cotransfected with VIB, a modified proviral construct that expresses a gRNA containing all the signals
306 needed for packaging [7]. Notably, V1B encodes a truncated assembly-incompetent Gag that does not
307 interfere with assembly of GagGFP expressed in trans. These constructs coexpressed *in trans*
308 (GagGFP/V1B, Fig 1A, Set II) have been well vetted in live imaging studies [8]. Use of this *in trans*
309 system also allowed us to express a single well-studied V1B genomic construct with different Gag
310 constructs. As expected, coexpression of GagGFP and V1B constructs resulted in the same VLP and
311 packaging-initiation-complex phenotypes observed for proviral constructs in Fig 1B (Fig S1A).

312 To test whether WT Gag forms the ~80S putative packaging initiation complex and larger ~500S
313 and ~750S complexes, we analyzed cell lysates coexpressing either WT Gag with V1B or G2A GagGFP
314 with the V1B genomic construct. In this experiment, we used a velocity sedimentation gradient that
315 allows resolution of early and late assembly intermediates [25,30]. Steady state levels of intracellular
316 Gag protein were similar for both WT and G2A, as were gRNA levels (Fig 4A). G2A Gag protein
317 formed only the ~10S and ~80S intermediates (as in Fig 3B); in contrast, at steady state, WT Gag protein
318 formed both of these complexes as well as the ~500S late assembly intermediate and ~750S completed
319 capsids (Fig 4B, WB), as shown previously [25,30,32]. Notably, nearly all the gRNA was in the ~80S
320 complex regardless of whether it was coexpressed with WT Gag or G2A Gag (Fig 4B, graph). IP with
321 αGFP revealed that both WT and G2A GagGFP were associated with V1B gRNA in the ~80S putative
322 packaging initiation complex (Fig 4C, graph). Additionally, αGFP IP showed that WT Gag was also
323 associated with V1B gRNA in the ~500S late assembly intermediate and the ~750S completed capsids,
324 both of which are formed by WT Gag but not targeting-defective G2A Gag (Fig 4C, graph). While COS-
325 1 cells were used in this experiment to avoid endocytosis of completed virus, as previously described
326 [25], similar results were obtained in human 293T cells expressing WT Gag that were harvested before
327 significant virus endocytosis had occurred (Fig S2A-C). Additionally, coIP results similar to those shown
328 in Fig 4C were obtained even in the absence of PuroHS treatment [25]; thus arguing that PuroHS
329 treatment allows us to study non-translating gRNA without altering the underlying biology. Thus, gRNA
330 is associated with WT Gag in the ~80S packaging initiation complex, the ~500S late intermediate, and the
331 ~750S completed immature capsid. These data are consistent with the ~80S complex being both the
332 packaging initiation complex and an early assembly intermediate, and with the ~500S complex being both
333 a late packaging intermediate and late assembly intermediate, as would be expected.
334 Thus far, we had shown that 1) both G2A Gag and WT Gag enter an ~80S RNA granule to form
335 the packaging initiation complex (Fig 3C, 4C; Fig S2C); 2) this ~80S packaging initiation complex
336 contains the RNA granule protein DDX6 (Fig 3F) and therefore appears to be an RNA granule; and 3)
337 WT Gag also forms a ~500S packaging/assembly intermediate containing Gag associated with gRNA

338 (Fig 4C; Fig S2C). Additionally, our previous coIP studies had shown that the ~80S and ~500S assembly
339 intermediates contain Gag in association with the cellular facilitators of assembly ABCE1 and DDX6
340 [11,14]. If the packaging initiation complex and late packaging intermediate are also assembly
341 intermediates, we would expect ABCE1 to be associated with gRNA in ~80S and ~500S
342 packaging/assembly intermediates formed by WT GagGFP. Indeed, αABCE1 immunoprecipitated V1B
343 gRNA from ~80S and ~500S intermediates formed by WT GagGFP (Fig S2D-F), thereby confirming that
344 these two complexes correspond to previously described ~80S and ~500S assembly intermediates derived
345 from RNA granules [11,14,25,33]. Thus, gRNA in the ~80S packaging initiation complex and the ~500S
346 packaging/assembly intermediate is associated with both WT Gag (Fig 4C, Fig S2C) and ABCE1 (Fig
347 S2F). These data support a model in which Gag enters an RNA granule that contains ABCE1, DDX6,
348 and gRNA to form the packaging initiation complex and remains associated with this granule as it
349 completes packaging and assembly.
350
351 **gRNA is present in RNA granule-like packaging intermediates in chronically infected T cells**
352 Previously, we showed that in human H9 T cells chronically infected with HIV (H9-HIV), Gag
353 associates with ABCE1 and DDX6 in ~80S and ~500S complexes by coIP [11]. Here we analyzed H9-
354 HIV cells on gradients and found that nearly all the non-nuclear non-translating gRNA is in ~80S and
355 ~500S complexes, and that gRNA in those complexes was associated with ABCE1 by coIP (Fig 4D-F).
356 Gag was also present in the ~80S and ~500S regions of the gradient, as expected (Fig 4E WB).
357 Interestingly, at steady state in H9-HIV lysate, the ratio of non-translating gRNA in 80S vs. 500S
358 intermediates was shifted towards more ~500S gRNA, compared to transfected COS-1 or 293T cells
359 (compare 4E, F to 4B,C and S2B, C). Together our findings demonstrate that in three different cell types,
360 including a more physiologically relevant HIV-infected human T cell line, gRNA is only associated with
361 Gag in RNA granule-derived assembly intermediates. Additionally, our findings raise the possibility that
362 formation of the late ~500S packaging intermediate occurs more efficiently in human T cells.
363

364 **Gag requires a gRNA-binding domain to enter the subset of granules containing genomic RNA**

365 Given the longstanding observation that the NC domain is required for association of Gag with
366 gRNA, we would expect NC to be critical for targeting Gag to gRNA-containing RNA granules. To test
367 this possibility, we took advantage of GagZip, in which NC is replaced with a heterologous leucine zipper
368 (LZ). GagZip has been used to demonstrate that NC has two functions during immature capsid assembly
369 - NC binds specifically to gRNA and also promotes oligomerization of Gag via non-specific RNA
370 association [21,24]. Because LZ promotes direct protein-protein interactions, it substitutes for the
371 oligomerization function of NC; thus, GagZip is assembly-competent and produces VLPs [22-24].
372 However, because LZ does not bind to RNA, these GagZip VLPs lack gRNA or other RNAs [23,24].
373 Interestingly, we found previously that GagZip forms the ~80S and ~500S ABCE1- and DDX6-
374 containing assembly intermediates [11,22], even though it produces VLPs that lack gRNA (Fig 1C).
375 These data raised the possibility that GagZip localizes to a broad class of ABCE1- and DDX6-containing
376 RNA granules, but fails to localize to the subset of these granules that contains gRNA because it lacks the
377 gRNA-binding NC domain.

378 Before testing this hypothesis, we first confirmed that GagZipGFP, like GagZip, produces VLPs
379 that lack gRNA when cotransfected with the V1B genome *in trans* (Fig S1A and 1C). In cells transfected
380 with WT Gag or GagZipGFP, and V1B *in trans* (Fig 1A, Set II constructs), both Gag proteins were
381 expressed at similar steady state levels as were their gRNAs (Fig 5A), and non-translating V1B gRNA
382 was primarily in the ~80S RNA granule in both cases (Fig 5B graph). WB confirmed that GagZipGFP
383 forms a prominent ~500S RNA granule-like assembly intermediate (Fig 5B WB). In addition, previously
384 we confirmed that GagZip also forms the ~80S RNA granule albeit at lower levels than for WT Gag (in
385 [22], see Fig 4 dark exposures and Fig 5). Notably, αGFP coimmunoprecipitated gRNA from ~80S and
386 ~500S fractions of cells expressing WT GagGFP (as in Fig 4C), but αGFP failed to coIP gRNA from any
387 fraction of the GagZipGFP gradient (Fig 5C, graph). Controls showed that αGFP immunoprecipitated
388 GagZipGFP protein as effectively as WT GagGFP from ~80S and ~500S fractions (Fig S3A), so the
389 failure to coIP gRNA cannot be attributed to reduced IP efficiency. These data argue that both WT Gag

390 and GagZip localize to ~80S RNA granules, but WT Gag stably associates with a subset of these RNA
391 granules that contains gRNA, while GagZip does not stably associate with the gRNA-containing subset of
392 these granules. Thus, the NC domain is required to form the packaging initiation complex, and acts by
393 directing Gag to the gRNA-containing granule subset. Moreover, our data suggest that a different region
394 of Gag (present in GagZip but not in MACA) is responsible for bringing Gag to a broader class of RNA
395 granules, most of which lack gRNA.

396 Finally, we asked whether we could restore targeting of GagZip to a gRNA-containing RNA
397 granule subset via a heterologous gRNA-binding domain. Because the V1B genomic RNA contains MS2
398 stem loops, we reasoned that the MS2 coat protein (MCP), which binds with high affinity to MS2 stem
399 loops (reviewed in [36]), could serve as a heterologous gRNA-binding domain if inserted into GagZip.
400 Therefore, we generated a construct, here called GagZipMCP, in which MCP is fused to the C-terminus
401 of GagZip. Additionally, we showed that GagZipMCP forms VLPs that contain the V1B genome, unlike
402 GagZip (Fig S3B). When VIB was cotransfected either with GagZip or GagZip MCP, to similar steady
403 state levels (Fig 5D), and analyzed on gradients, gRNA was found largely in ~80S granules in both cases
404 (Fig 5E). However, αGFP coimmunoprecipitated gRNA from ~80S and ~500S fractions formed by
405 GagZip MCP GFP, but not from the corresponding complexes formed by GagZip, analyzed in parallel
406 (Fig 5F). Thus, fusion to MCP, a heterologous gRNA-binding domain, redirected GagZip to the specific
407 subset of RNA granules that contains gRNA, resulting in formation of the gRNA-containing ~80S
408 packaging initiation complex and ~500S intermediates. These data confirmed that a gRNA-binding
409 domain is required to target Gag to the subset of granules containing gRNA.

410

411 ***In situ* studies confirm that assembling Gag colocalizes with the RNA granule proteins**

412 Next we asked whether we can confirm the presence of Gag in RNA granules in intact cells by
413 visualizing sites where Gag colocalizes with DDX6 or ABCE1 using the proximity ligation assay (PLA).
414 PLA produces fluorescent spots at sites where two proteins are within 40 nm of each other *in situ*.
415 Briefly, this method uses antibodies conjugated to complementary oligonucleotide probes to detect two

416 proteins of interest; the probes anneal only when within the 40 nm range, leading to a rolling circle
417 amplification product that is recognized by a fluorophore-conjugated oligonucleotide [37](Fig 6A). If
418 assembling Gag enters RNA granules containing ABCE1 and DDX6 as indicated by our biochemical
419 studies, then the assembling Gag constructs (WT Gag, G2A Gag, and GagZip) should produce abundant
420 Gag-DDX6 and Gag-ABCE1 PLA spots relative to assembly-incompetent MACA Gag, which fails to
421 enter granules (Fig 3A-C) and does not coIP with DDX6 or ABCE1 [11,25]. To test this, 293T cells were
422 transfected with WT vs. mutant provirus (Fig 1A, Set I) and analyzed for Gag-DDX6 or Gag-ABCE1
423 colocalization by PLA. Concurrent Gag IF (Fig 6A) allowed us to confirm that the majority of PLA spots
424 were observed in Gag-expressing cells, and to choose fields for quantitation with comparable Gag levels.
425 For cells expressing WT Gag, G2A Gag, or GagZip, quantified fields contained ~50 Gag-DDX6 PLA
426 spots per cell (Fig 6 B, C), three-fold more than for cells expressing MACA. Similar results were
427 observed for Gag-ABCE1 PLA spots (Fig 7). Some PLA background was expected in cells transfected
428 with MACA provirus, given that DDX6 and ABCE1, like MACA (Fig 3B), are found in the ~10S fraction
429 and could therefore be in proximity outside of granules. Thus, PLA appears to identify DDX6- and
430 ABCE1-containing RNA granules co-opted by Gag. Moreover, PLA confirms our biochemical studies in
431 which we showed that WT Gag, G2A Gag, and GagZip target to ABCE1- and DDX6-containing RNA
432 granules (Fig 3 - 5), as well as previous quantitative immunoelectron microscopy studies showing the
433 colocalization of ABCE1 and DDX6 with assembling Gag [11,25,30,33].
434

435 **Sites of Gag-DDX6 and Gag-ABCE1 interaction *in situ* are far more numerous than P bodies**

436 While DDX6 is a marker of P bodies, as shown by intense labeling of P bodies by DDX6 IF [31],
437 DDX6 is also found in smaller RNA granules [11]. Given that cells typically contain fewer than ten P
438 bodies [38], our finding that each Z stack image contains ~50 Gag-DDX6 PLA spots (Fig 6) suggested
439 that granules containing Gag and DDX6 are far more numerous than P bodies. To test this possibility
440 directly, we analyzed 293T cells for G2A Gag-DDX6 PLA spots with concurrent DDX6 IF, to allow
441 detection of PLA spots and P bodies in the same fields (Fig 8A). G2A provirus was used here because

442 G2A Gag is arrested as the DDX6-containing ~80S packaging initiation complex (Fig 3B, C, F,); thus,
443 most G2A Gag-DDX6 PLA spots likely represent packaging initiation complexes. Each Z stack image
444 from G2A-expressing cells displayed an average of 56 Gag-DDX6 PLA spots, but only one P body by
445 DDX6 IF (Fig 8B, C). Thus, packaging initiation complexes are far more numerous than P bodies.
446 Interestingly, DDX6 IF with high gain revealed a diffuse, low-intensity, granular DDX6 signal (Fig 8C
447 insets), in addition to the bright DDX6 positive P body foci, in both G2A-expressing and mock cells. The
448 low-intensity DDX6 signal could correspond to DDX6-containing ~80S RNA granules, some of which
449 are co-opted to form packaging initiation complexes.
450
451 **Association of gRNA with DDX6 at the PM *in situ* upon expression of WT Gag but not GagZip**
452 Our biochemical studies showed that WT Gag remains associated with the co-opted RNA granule
453 during late stages of assembly (Fig4), suggesting that assembling Gag takes the co-opted gRNA-
454 containing granule to PM sites of budding and assembly. In contrast, we found that GagZip associates
455 with RNA granule proteins at late stages of assembly, but not with the subset of RNA granules that
456 contains gRNA (Fig 5). Thus, we would expect *in situ* approaches to reveal DDX6-containing RNA
457 granules to be present at WT Gag or GagZip PM sites of assembly; however, the granules co-opted by
458 WT Gag should also contain gRNA, while the granules co-opted by GagZip should contain DDX6 but no
459 gRNA. Previously, we used quantitative immunoelectron microscopy (IEM) to demonstrate that RNA
460 granule proteins (ABCE1 and/or DDX6) are recruited to PM sites of WT Gag and GagZip assembly
461 [11,22,33]; however, these studies did not assess whether gRNA was associated with these granules.
462 Here we used quantitative IEM with double labeling for gRNA and DDX6 to ask whether gRNA is
463 associated with RNA granules at PM sites of assembly for WT Gag vs. GagZip *in situ* (Fig 9). HeLa cells
464 stably expressing MCP fused to GFP (HeLa-MCP-GFP cells) were transfected with V1B genomic
465 constructs encoding MS2 binding sites and Gag *in cis* (Fig 9A; Fig 1A, Set IV constructs; phenotypes
466 confirmed in Fig S1B). Sections were labeled with αDDX6 (large gold) to mark RNA granules, and with
467 αGFP to mark MCP-GFP-tagged gRNAs (small gold). PM assembly sites, defined by the presence of

468 membrane deformation consistent with budding, were quantified and scored for gRNA labeling, DDX6
469 labeling, and double labeling (Fig 9B, C; Table S1). When all sites of DDX6 labeling at PM assembly
470 sites were quantified, similar high levels of DDX6 labeling were observed at both WT and GagZip PM
471 assembly sites (56% vs. 40% of all WT vs. GagZip PM assembly sites displayed DDX6 labeling,
472 respectively, $p>0.01$; shown as total DDX6+ in Fig 9B; shown as D+ tot in Table S1). Notably,
473 quantitation of all sites of gRNA labeling at the PM revealed that gRNA was significantly more common
474 at WT assembly sites relative to GagZip PM assembly sites (64% vs. 20% of all WT vs. GagZip PM
475 assembly sites displayed gRNA labeling, respectively, $p<0.005$; shown as total gRNA+ in Fig 9B; shown
476 as g+ Tot in Table S1). Our most striking results were obtained upon quantitation of PM assembly sites
477 that were double labeled for DDX6 and gRNA. Abundant gRNA and DDX6 double labeling was
478 observed at WT PM assembly sites, but not at GagZip PM assembly sites (33% vs. 5% of all WT vs.
479 GagZip PM assembly sites, respectively, displayed double labeling, $p<0.005$; shown as gRNA+/DDX6+
480 in Fig 9B; shown as g+D+ in Table S1). As expected, the assembly-incompetent MACA formed very
481 few early or late PM assembly sites, unlike WT and GagZip. The same patterns were observed when
482 early and late assembly sites were analyzed separately (Table S1). Thus, IEM analysis of PM assembly
483 sites supports our conclusion that both WT and GagZip co-opt RNA granules during packaging and
484 assembly, but only the RNA granules co-opted by WT Gag also contain gRNA. Moreover, these
485 quantitative IEM studies (Fig 9) along with our PLA studies (Fig 6-8) provide *in situ* validation, in the
486 absence of PuroHS treatment, of our biochemical studies.
487
488 **Discussion:**
489 It is increasingly evident that, within cells, the fate of an RNA is determined in large part by its
490 associated cellular proteins [39]. For this reason, it will be important to determine whether HIV-1
491 packaging initiation occurs in the context of cellular RNA binding proteins. Here we showed that almost
492 all of the non-nuclear, non-translating gRNAs in HIV-1 expressing cells are found in large RNPCs
493 (>30S). Moreover, a subclass of these RNPCs, identified as ~80S host RNA granules, is co-opted to

494 form the packaging initiation complex. We identified this ~80S host RNA granule as the packaging
495 initiation complex by demonstrating that it is the only gRNA-containing complex formed by the G2A
496 Gag mutant, which arrests at packaging initiation (Fig 3C). We also demonstrated that the RNA-granule-
497 derived ~80S packaging initiation complex contains the host enzymes ABCE1 and DDX6, and is found in
498 HIV-1-infected human T cells. Additionally, our studies revealed that Gag does not require a gRNA-
499 binding domain to stably associate with ~80S host RNA granules, but does require a gRNA-binding
500 domain to stably associate with the subset of these granules that contains gRNA. Finally, we showed that
501 packaging initiation complexes are far more numerous than P bodies *in situ*; thus, packaging initiation
502 complexes are not equivalent to P bodies, consistent with our finding that packaging initiation complexes
503 correspond to smaller ~80S RNA granules.

504 Based on our findings, we propose the following model for initiation of gRNA packaging (Fig
505 10). In the cytoplasm, gRNA is either in translating complexes or in non-translating host RNPCs of
506 ~30S-80S. WT Gag uses a two-step mechanism to co-opt gRNA-containing granules to form the ~80S
507 packaging initiation complex. One of these two steps involves an event that is independent of gRNA
508 binding, and localizes WT Gag to a broad class of ABCE1- and DDX6-containing ~80S RNA granules.
509 The second step involves a gRNA-binding-dependent event that localizes WT Gag to the subset of these
510 granules that contains gRNA. In the case of WT Gag, targeting, packaging, and late stages of
511 multimerization continue in association with this RNA granule, leading to formation of the ~500S
512 packaging/assembly intermediate and the ~750S completely assembled capsid, both of which contain
513 gRNA. The packaging initiation complex and the ~500S packaging/assembly intermediate contain
514 ABCE1 and DDX6, two host enzymes that facilitate virus assembly [11,14] and that may distinguish this
515 subclass of granules. In contrast, the intracellular ~750S completed capsid has dissociated from the RNA
516 granule [11,14,32,33] and subsequently completes budding and release. Three caveats to this model
517 should be noted. First, how gRNA enters the host RNA granules is not known. Most likely, gRNA-
518 containing RNPCs are generated during transcription and undergo successive rounds of remodeling
519 during nuclear export and in the cytoplasm to form ~80S non-translating RNA granules and translating

520 complexes in the cytoplasm. Secondly, while we refer to complexes in the ~80S region as a single ~80S
521 complex for simplicity, future studies will be required to determine if complexes in this region are
522 homogenous or display some heterogeneity. Thirdly, while our coIP studies demonstrate that Gag is
523 associated with gRNA in the packaging intermediates, we do not know whether Gag and gRNA make
524 direct contact with each other in these complexes. Based on studies by others [40], we speculate that Gag
525 proteins make direct contact with only a few regions of gRNA in the ~80S packaging initiation complex,
526 but contact many more regions of the gRNA in the ~500S late packaging intermediate.

527 A stunning finding of this study is that the packaging initiation complex has an S value similar to
528 the eukaryotic ribosome, a large RNPC containing four RNAs and 79 cellular proteins. Interestingly,
529 studies by others are consistent with this finding. Specifically, the reported diffusion coefficient of ~70S
530 bacterial ribosomes (0.04 μm^2 sec^{-1}; [41] is similar to the ~0.07 and 0.014 to μm^2 sec^{-1} diffusion
531 coefficients reported for cellular subpopulations of HIV-1 gRNA [9] and Gag [10], respectively. Our
532 finding that the packaging initiation complex is ~80S suggests that this complex contains numerous
533 additional components, since Gag is likely a dimer or small oligomer at this stage [7], and a Gag dimer on
534 its own would be ~5S. Previously we showed that two other viral proteins are present in assembly
535 intermediates – HIV-1 GagPol [33] and Vif [14]; however, it is likely that host components account for
536 much of the molecular mass of the ~80S packaging initiation complex. Here we showed that the
537 packaging initiation complex contains at least two host enzymes that are known to facilitate HIV-1 capsid
538 assembly, ABCE1 and DDX6 [11,14]. In addition, our earlier studies demonstrated that the RNA granule
539 proteins AGO2 and DCP2 are also present in these RNA-granule-derived assembly/packaging
540 intermediates [11]. Further studies will be required to define the exact proteome and RNAome of the
541 ~80S packaging initiation complex and determine whether this complex contains other RNA binding
542 proteins such as Staufen1, which plays a role in HIV-1 packaging and assembly [42-44], and MOV10,
543 which is packaged by HIV-1 and modulates virus infectivity [45,46].

544 Importantly, using PLA we showed that although the packaging initiation complex contains the P
545 body marker DDX6, it is not equivalent to a P body, and corresponds instead to granules that are much

546 smaller than P bodies (Fig 8). The PLA findings correspond well with our biochemical findings, which
547 suggest that the ~80S granule should be similar in size to the ~80S ribosome, which is ~25 nm in
548 diameter [47]; thus the DDX6-containing ~80S granule should be four to twelve times smaller than
549 DDX6-containing P bodies, which range from 100 – 300 nm in size [48]. These findings are also
550 consistent with an earlier study showing that Gag and gRNA are not found in P bodies [49]. Note,
551 however, that the co-opted ~80S RNA granules could represent P body subunits or could exchange with P
552 bodies. Thus, we cannot rule out the possibility that the packaging initiation complex is occasionally
553 found in P bodies, perhaps explaining an earlier report [50]. Future studies will need to compare the
554 components of P bodies with those of the ~80S RNA granule that contains the vast majority of the gRNA
555 in HIV-1 infected cells. However, it is worth noting that in the case of the Ty3 yeast retrotransposon,
556 which is distantly related to HIV-1, assembling Ty3 Gag and its packaged Ty3 RNA are found in large
557 clusters that contain the yeast DDX6 homologue dhh1 [51,52]; moreover knockdown and mutational
558 analyses indicate a role for RNA granule proteins in formation of functional Ty3 particles in yeast
559 [53,54]. Similarly, we had previously shown using siRNA knockdown that DDX6 promotes immature
560 HIV-1 capsid assembly, and can be rescued by a siRNA resistant WT DDX6 but not a ATPase-defective
561 DDX6 mutant [11]. While others have not observed that DDX6 is required for HIV-1 capsid assembly
562 [49], this could be because other helicases in the co-opted RNA granule can substitute for DDX6 in some
563 cell types or when given enough time to undergo upregulation.
564 Our identification of the two-step mechanism for stable association of Gag with gRNA-
565 containing ~80S granules resulted from the observation that an assembling Gag that lacks a gRNA-
566 binding domain (GagZip) targets to RNA granules that lack gRNA, but can be redirected to gRNA-
567 containing granules by addition of a heterologous gRNA-binding domain (Fig 5C, F; [11,22]). Thus, it
568 appears that most ~80S RNA granules contain cellular RNAs, with only a small subset containing gRNA.
569 Notably, a key difference between MACA, which fails to enter RNA granules, and GagZip, which enters
570 ABCE1- and DDX6-containing granules that lack gRNA, is that GagZip has a heterologous
571 oligomerization domain (LZ) that substitutes for NC, a domain in WT Gag that mediates oligomerization.

572 Together, these data argue that two events are required for stable association of Gag with gRNA-
573 containing granules, with one being the aforementioned poorly understood, NC-independent step in
574 which oligomerization-competent Gag targets to a large class of ~80S ABCE1- and DDX6-containing
575 RNA granules, most of which lack gRNA; and the other being a gRNA-binding step, dependent on NC or
576 a heterologous gRNA-binding domain, that enables stable association with a gRNA-containing subset of
577 these granules.

578 Our finding that, in WT Gag, the NC domain is required for packaging initiation complex
579 formation fits with decades of studies showing the importance of NC in packaging. At the same time, our
580 data argue for a new view of packaging. To date, packaging studies have not specified where or how Gag
581 associates with gRNA; we fill this gap by providing evidence that packaging is initiated within a unique
582 host RNPC that contains RNA granule markers and nearly all the packageable gRNA. Interestingly,
583 sequesteration of gRNA within unique host RNA granules could be both beneficial and problematic for
584 the virus. On the one hand, gRNA sequestration is likely to be highly advantageous to the virus - it could
585 allow gRNA to evade detection by the host immune system, create a site where assembling Gag can be
586 concentrated, and provide Gag access to RNA helicases that could facilitate displacement of host RNA
587 binding proteins from gRNA allowing them to be replaced with Gag. On the other hand gRNA
588 sequestration creates a dilemma for the virus in that it puts gRNA in a different subcellular compartment
589 (the RNA granule) than newly translated Gag, which is found either associated with translating ribosomes
590 or in the soluble compartment. To solve this problem, Gag appears to have evolved a mechanism for
591 localizing to a subclass of mRNA-containing host RNA granules, which in turn allows efficient NC-
592 dependent localization to the small subset of these granules that contains gRNA. Future studies will need
593 to further test this model and address how both the cell and the virus utilize this unique class of RNA
594 granules.
595
596
597

598 **Materials and Methods:**

599 **Plasmids and cells**

600 Four types of expression systems were utilized in this study. Proviruses (Set I in Fig 1A) are
601 from the LAI strain and were described previously [22,33,55]. These have native HIV-1 sequences and
602 the HIV-1 LTRs. Proviruses were transfected into COS-1 or 293T cells (both obtained from the
603 American Type Culture Collection (ATCC), Manassas, VA) as indicated for some biochemical studies
604 and all PLA studies. For other biochemical studies, we used an *in trans* expression system in which the
605 genome is provided by V1B, a modified proviral plasmid that expresses an RNA encoding an assembly-
606 incompetent truncated *gag* gene, all cis-acting packaging signals, full-length *tat*, *rev*, and *vpu* genes, and
607 24 MS2 stem loops that bind to MCP [8]. V1B was transfected into COS-1 or 293T cells with WT and
608 mutant SynGagGFP constructs (here referred to as GagGFP constructs) provided *in trans*, where
609 indicated. V1B and the codon optimized SynGagGFP WT and G2A constructs (Set II in Fig 1A) were
610 provided by P. Bieniasz (Rockefeller University, New York, N.Y.) and were utilized in previous live
611 imaging studies [8] and coIP studies [7]. Additional GagGFP constructs (MACA and GagZip) were
612 generated from the WT SynGagGFP construct. For G2A, the glycine in position 2 of Gag was converted
613 to an alanine by via site-directed mutagenesis, as described previously [33]; for GagZip, a leucine zipper
614 was inserted in place of nucleocapsid using Gibson assembly, as described previously [22]. For IEM
615 experiments, HeLa cells that express MCP-NLS-GFP as described previously [8] were obtained from P.
616 Bieniasz (Rockefeller University, New York, N.Y.). To ensure that all HeLa-MCP-GFP cells expressed
617 both Gag and genome following transfection, V1B constructs expressing WT and mutant Gag *in cis* were
618 generated by inserting relevant Gag coding regions from HIV-1 proviruses (Set I in Fig 1A) into V1B
619 constructs containing MS2 binding sites via Gibson assembly to generate Set IV constructs in Fig 1A.
620 Oligos used for site-directed mutagenesis and Gibson assemblies are available upon request.

621 H9 cells (ATCC) were used to generate H9-HIVpro- cells (Set III in Fig 1A) by infection with
622 virus. Plasmid used for virus production was generated by inserting three protease inactivation mutations
623 into a previously described HIV-1 provirus, LAI strain, that encodes deletions in *env* and *vif*, a frameshift

624 in *vpr*, and substitution of *nef* with a puromycin resistance gene, as described previously [11]. 293T cells
625 were transfected with this plasmid to produce virus, and H9 cells were infected with this virus and
626 maintained under puromycin selection as described previously [32].
627 COS-1 and 293T cells were maintained in DMEM (Life Technologies) with 10% FBS. H9 cells
628 were maintained in RPMI (Life Technologies) with 10% FBS under puromycin selection. HeLa-MCP-
629 GFP cells were obtained from P. Bieniasz and were maintained in DMEM with 10% FBS, and
630 periodically subjected to blastocidin selection.
631
632 **Transfection, IP, and WB**
633 COS-1, 293T, or HeLa-MCP-GFP cells were transfected with 1-5µg DNA using
634 polyethylenimine (Polysciences, Warrington, PA). Cell lysates were harvested in 1X Mg^{+2}-containing
635 NP40 buffer (10 mM Tris-HCl, pH 7.9, 100 mM NaCl, 50 mM KCl, 1 mM MgCl, 0.625% NP40) in the
636 presence of freshly prepared protease inhibitor cocktail (Sigma, St Louis, MO) and RNaseOUT
637 (Invitrogen). Where indicated, lysates were treated with 1 mM puromycin HCl (Invitrogen) for 10 min at
638 26°C followed by 0.5M KCl for 10 min at 26 °C. Lysates were analyzed by WB or RT-qPCR, or
639 subjected to IP as described below. Alternatively, lysates were analyzed by velocity sedimentation, as
640 described below, and gradient fractions were then analyzed by WB, RT-qPCR, or IP.
641 Except where indicated, lysates or gradient fractions were subjected to IP with affinity purified
642 antibody to ABCE1 [14], HIV immunoglobulin NIH AIDS Reagents Catalog #3957, from NABI and
643 NHLBI), a monoclonal to GFP (Roche), or an antibody to DDX6 (#461, Bethyl Laboratories,
644 Montgomery, TX), using protein G-coupled Dynabeads (Life Technologies). IP eluates were analyzed by
645 SDS-PAGE, followed by western blot (WB) using the primary antibodies described above or a
646 monoclonal antibody directed against HIV-1 Gag p24 (HIV-1 hybridoma 183-H12-5C obtained from
647 Bruce Chesebro through the AIDS Reagent Program Division of AIDS, NIAID, NIH), followed by an
648 HRP-conjugated anti-human IgG secondary antibody (Bethyl Laboratories, Montgomery, TX), or an
649 HRP-conjugated anti-mouse-IgG_1 (Bethyl Laboratories) or anti-mouse-IgG or anti-rabbit secondary

650 antibody (Santa Cruz Biotechnology, Dallas, TX). In Suppl. Fig 3A, IPs were subjected to WB with HIV
651 immune globulin (provided by NABI and NHLBI, catalog no. 3957 in the AIDS Reagent Program
652 Division of AIDS, NIAID, NIH) for detection of Gag. WB signals from IP eluates were detected using
653 Pierce ECL substrate (Thermo Fisher Scientific) with Carestream Kodak Biomax Light film. For
654 detection of Gag in total cell lysates, velocity sedimentation fractions, and membrane flotation fractions,
655 WBs were performed as described above, or using antibodies conjugated to infrared dyes (LI-COR
656 Biosciences, Lincoln, NE). Quantification of Gag bands on film was performed using Image J software
657 or LI-COR Odyssey software.
658
659 **RNA quantification**
660 Transfected cells or VLPs were harvested as described above. Where indicated lysates were analyzed by
661 velocity sedimentation and or IP, as described above except that IPs for gRNA quantification were
662 washed four times in detergent buffer and once in non-detergent buffer. For total cell lysate analysis,
663 aliquots corresponding to ~5 x10^3 COS-1 cells, 2 x10^4 293T cells and 8.5 x10^3 HeLa-MCP-GFP cells
664 were used. For VLP analysis, aliquots corresponding to VLPs from 1 x10^5 293T cells and 1.5 x10^4 HeLa-
665 MCP-GFP cells were used. For gradient analysis, ~4 x10^5 COS-1 cells, 2 x 10^6 203T cells, or 6 x 10^5 H9
666 cells were analyzed on a single 5 ml gradient. Aliquots of gradient fractions or gradient IP fractions were
667 treated with proteinase K (Sigma) at a final concentration of 150 µg/ml in 0.1% SDS, followed by total
668 RNA extraction by using Trizol (Ambion). RNA was precipitated with isopropanol, extracted with BCP
669 (Molecular Research Center), pelleted at 12,000xg for 15 min at 4°C, and the RNA pellet was subjected
670 to DNase I (Invitrogen) treatment (2 u per 50 ul reaction). The iScript Advanced cDNA synthesis kit
671 (Bio-Rad) was used to generate cDNA from 10% of the RNA using random hexamer primers at 42°C for
672 30 min, followed by heat inactivation. An aliquot of cDNA (2.9%) was used for qPCR using SYBR
673 Green (Bio-Rad) to determine RNA copy number. For HIV-1 gRNA qPCR, we used the following oligos
674 that target bp 162 to 269 within the Gag open reading frame in HIV-1 LAI (at the end of MA and start of
675 CA) and result in a 108 bp amplicon: 5'-AGAAGGCTGTAGACAAATACTGGG-3' (forward); 5'-

676 TGATGCACACAATAGAGGGTTG-3' (reverse). These oligos detect the full length HIV-1 provirus as
677 well as the V1B genomic construct, but do not recognize the GagGFP constructs that were transfected *in*
678 *trans*, as expected since the GagGFP constructs were codon-optimized. To determine the copy number of
679 other RNAs, we used the following qPCR oligos: Tat mRNA, 5'-TCT ATC AAA GCA ACC CAC CTC-
680 3' (forward) and 5'- CGT CCC AGA TAA GTG CTA AGG-3' (reverse); 28S rRNA, 5'-CCC AGT GCT
681 CTG AAT GTC AA-3' (forward) and 5'-AGT GGG AAT CTC GTT CAT CC-3' (reverse); GAPDH
682 mRNA, 5'-AGG TCA TCC CTG AGC TGA AC-3' (forward) and 5'-GCA ATG CCA GCC CCA GCG
683 TC-3' (reverse); and 7SL RNA 5'-GCT ATG CCG ATC GGG TGT CCG-3' (forward) and 5'-TGC AGT
684 GGC TAT TCA CAG GCG-3'(reverse). All qPCR samples were analyzed in duplicate using the MyiQ
685 RT-PCR detection system and iQ5 software (Bio-Rad). Amplicons corresponding to region amplified by
686 qPCR were used to generate standard curves. Duplicate nine-point standard curves were included on
687 every qPCR plate, ranging from 10^1 copies to 10^8 copies, with a typical efficiency of ~90% or greater and
688 an R^2 of 0.99. Standard curves were able to detect 10 copies per reaction but not 1 copy, thereby setting
689 the detection threshold at 10 copies per reaction, which was equivalent to ~1000 copies per 1000 cells for
690 inputs and total gRNA from gradient fractions, ~100 copies per 1000 cells for IPs from total cell lysates
691 or gradient fractions, and ~50 copies per 1000 cells for VLPs. Minus RNA controls were included in
692 each experiment and were always zero. RT minus controls were also included in each experiment and
693 ranged from 0 - 100 copies per reaction. Mock transfected VLP controls were used to set the baseline in
694 graphs and ranged from 1 - 1000 copies per 1000 cells. For IPs, nonimmune gRNA copy number was
695 analyzed in parallel and was typically 1-2 logs lower than IPs.
696 Quantification of the total cell number used in each experiment allowed us to represent all qPCR
697 data as number of gRNA copies per 1000 cells, except for Fig 2 data which is presented as % of total
698 gRNA to allow comparison across different RNAs. Log scales were used to display all VLP, which
699 exhibit large differences. Linear scales were used for IP data, which exhibit smaller differences. Note
700 that differences in transfection efficiency resulted in a range of total gRNA copies per 1000 cells between
701 experiments; likewise, IP efficiency also varied between experiments. Thus the exact number of gRNA

702 copies immunoprecipitated in fractions from different experiments varied considerably, but the pattern
703 did not.
704
705 **Analysis of VLP production**
706 Supernatants of COS-1 or HeLa-MCP-GFP cells, transfected as described above, were centrifuged at
707 2000 rpm (910 x *g*) for 10 min at 4°C, filtered (0.45 μm) to remove remaining cells, and purified through
708 a 30% sucrose cushion in an SW60Ti rotor at 60,000 rpm (370,000 x *g*) for 30 min at 4°C, as described
709 previously [22].
710
711 **Velocity Sedimentation**
712 Transfected COS-1 cells or 293T cells were harvested at 36 h or 15 h post-transfection,
713 respectively, as described above and diluted into 1X NP40 buffer (10 mM Tris acetate pH 7.4, 50 mM
714 KCl, 100 mM NaCl, 0.625% NP-40). For each sample, 120 µl lysate was layered on a step gradient. To
715 resolve complexes of ~10S to ~150S (Fig 2, 3), step gradients were prepared from 5%, 10% 15%, 20%,
716 25%, and 30% sucrose in NP40 buffer without MgCl (10 mM Tris-HCl, pH 7.9, 100 mM NaCl, 50 mM
717 KCl, 0.625% NP40) and subjected to velocity sedimentation in a 5 ml Beckman MLS50 rotor at 45,000
718 rpm (162,500 x *g*) for 90 min at 4°C. To resolve from ~10S to ~750S, step gradients were prepared from
719 10%, 15%, 40%, 50%, 60%, 70%, and 80% sucrose in NP40 buffer without MgCl, and subjected to
720 velocity sedimentation in a 5 ml Beckman MLS50 rotor at 45,000 rpm (162,500 x *g*), for 45 min at 4°C.
721 Gradients were fractioned from top to bottom, and aliquots were analyzed by WB, IP, and/or RT-qPCR as
722 described above. S values were determined, using a published equation[56] and S value markers, as
723 described previously [34].
724
725 **Proximity Ligation Assay**
726 293T cells were plated into 6-well dishes containing coverslips with Grace Biolabs CultureWell
727 silicone chambers (Sigma-Aldrich) attached to create four chambers on each coverslip. Cells were

728 transfected with 3 µg of plasmid per well and 16.5 hours later were fixed for 15 minutes in 4%
729 paraformaldehyde in PBS pH 7.4, permeabilized in 0.5% saponin in PBS, pH 7.4 for 10 minutes, and
730 blocked in Duolink blocking solution (Sigma-Aldrich) at 37°C for 30 min. Cells were incubated in
731 primary antibody (described under IP methods above), followed by Duolink reagents (Sigma-Aldrich):
732 oligo-linked secondary antibody, ligation mix, and red or green amplification/detection mix, with washes
733 in between, as per the Duolink protocol. For concurrent IF, cells were incubated for 15 minutes at RT
734 with 1:1000 Alexafluor 594 anti-mouse or Alexafluor 488 anti-rabbit secondary antibody following the
735 final PLA washes. Cover slips were mounted using Duolink In Situ Mounting Media with DAPI, sealed
736 to the glass slides with clear nail polish, allowed to dry for 24 h at RT, and stored at -20°C. Imaging was
737 performed with a Zeiss Axiovert 200M deconvolution microscope using Zeiss Plan-Apochromat 63X/
738 aperture 1.4 objective with oil immersion, with AxioVision Rel. 4.8 software. For quantification, five
739 fields containing at least three IF or PLA positive cells were chosen at random and imaged using identical
740 exposure times for the red and green channels (red/green exposures were 1 second/1.5 seconds for Figure
741 6, 2 seconds/1 second for Figure 7, and 40 milliseconds/250 milliseconds for Figure 8). Images were
742 captured as ten 1-µm Z-stacks centered on the focal point for the PLA. Images were deconvolved using
743 the AxioVision software, then exported as .tif files, and Image J was used to outline Gag-positive cells in
744 each field. Within those positive cells, the central Z-stack image was used to count PLA "spots", and
745 quantify IF intensity where indicated, using Image J. PLA spot number for each field was then
746 normalized to the average IF intensity within that field, and the results were plotted with error bars
747 representing the SEM for five fields. For Figure 8, Gag-DDX6 PLA was done with either Gag IF or
748 DDX6 IF, and the PLA paired with Gag IF was used for PLA spot quantitation to exclude background
749 spots in Gag-negative cells, whereas the PLA paired with DDX6 IF was used for P body
750 quantitation. For the sample field images used in each figure, after imaging, the red channel gain was
751 increased proportionally (to 3 in Figure 6, to 11 in Figure 7, and to 7 in Figure 8) in the AxioVision Rel.
752 4.8 software for all conditions, to allow better display of red spots in final figures. The same was done for
753 the green channel gain (increased to 7) to display smaller DDX6 granules in the Fig 8D insets. Images

754 were imported in 8-bit color into Adobe Illustrator to create the final figure layout, without further
755 adjustments to color balance or gamma correction.
756
757 **Quantitative IEM**
758 HeLa-MCP-GFP cells were transfected with the indicated constructs. Cells were harvested at 24
759 h post-transfection in fixative (3% paraformaldehyde, 0.025% glutaraldehyde in 0.1 M phosphate buffer,
760 pH 7.4), pelleted, and subjected to high pressure freezing using the Leica EMPACT2, followed by freeze
761 substitution. Samples were infiltrated overnight with LR White embedding resin (London Resin
762 Company Ltd, Reading, Berkshire, England) in ethanol, changed to straight LR White, embedded in
763 gelatin capsules (Electron Microscopy Sciences (EMS), Hatfield, PA, USA), and cured overnight in a UV
764 light cryo-chamber at 4° C. Sections (~50 nm) were placed on grids, treated with 0.05 M glycine for 20
765 min at RT, rinsed in PBS, blocked for 45 min with 1% bovine serum albumin (EMS), and washed in PBS
766 with 0.1% bovine serum albumin-C (BSA-C) (EMS). For immunogold double-labeling, a previously
767 described peptide-specific antiserum directed against DDX6 was affinity purified, desalted, and
768 concentrated [11]. Grids were blocked in 0.5% BSA-C, then incubated with primary antibody to DDX6
769 (0.1 mg/ml in 0.5% BSA-C), followed by goat a-rabbit F(ab')2 fragment secondary antibody conjugated
770 to 15nm gold particles (EMS), with washes after each step. Grids were then labeled with the second
771 primary, mouse antibody to GFP (Roche) at 0.2 mg/ml in 0.1% BSAC with 0.002% Tween, followed by
772 goat a-mouse F(ab')2 fragment conjugated to 6 nm gold particles (EMS). Fixation, negative staining,
773 imaging with the JEOL-1400 transmission electron microscope, and image acquisition have been
774 described previously [11].
775 For quantification, images were acquired for ten cells from each of the three groups, with the goal
776 being to analyze similar total PM lengths in each group. Cells were chosen randomly, but excluded for
777 the WT and GagZip groups if they had fewer than ten particles at the PM visible at low power. Images
778 encompassed the area of each cell that contained PM assembly sites, with images obtained for ~250 μm
779 of PM total per group. The number of assembly sites analyzed within this ~250 μm of PM are not

780 equivalent since the number of assembly sites depends on VLP phenotype and kinetics. A total of 760
781 WT events and 409 GagZip events were analyzed, but are shown as number of sites per 25 μm PM per
782 cell in Table 1. Each PM assembly site was scored as genome positive (g+), DDX6+ (D+), or double-
783 labeled (g+D+). The following definitions were used for image analysis: early PM assembly sites were
784 defined as displaying curvature at the membrane but with < 50% of a complete bud; late assembly sites"
785 at the PM were defined as displaying curvature but with ≥ 50% of a complete bud. If early or late sites
786 contained two or more small gold particles within the full circle defined by the bud, they were scored as
787 g+. If these sites contained one or more large gold particles within a 150 nm perimeter outside the full
788 circle defined by the bud (roughly the size of an RNA granule plus space to account for the antibodies and
789 gold particle bound to an antigen at the periphery of such a granule), then they were scored as D+. Table
790 1 shows the average number of early, late, and early+late PM assembly events per 25 μm of PM per cell
791 (n=10 cells +/- SEM), along with the breakdown of how many of these events were g+ (total vs. single-
792 labeled), D+ (total vs. single-labeled), or g+D+ (double-labeled). In italics are g+, D+, and g+D+ per 25
793 μm of PM per cell as a percentage of the total for each group. Significance was determined on percentage
794 data using a two tailed t-test; not significant was defined as $p > 0.01$. Labeling of early+late events as a
795 percent of total early+late events is also shown in graphical form in Fig 8B. As described previously
796 [22], the sensitivity of IEM for capturing colocalization is limited by a number of factors including the
797 fact that the 50 nm sections only capture ≤ 50% of a single capsid, which has a diameter of ~100-150 nm.
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986 **Figure Captions**:

987 **Fig 1. Gag constructs: diagrams and phenotyopes**

988 **A)** Diagram of the different *cis* and *trans* expression systems used (Sets I – IV). Only WT constructs are
989 shown here, with mutant constructs diagrammed in later figures**. Set I** consists of WT (and mutant) HIV-
990 1 proviruses (pro-, delta env). **Set II** consists of codon-optimized WT (and mutant) Gag constructs fused
991 to GFP (GagGFP), transfected with a modified genomic construct (V1B) provided *in trans*. **Set III**
992 consists of proviruses from Set I in which the *nef* gene was replaced with a puromycin resistance gene
993 (PURO-R). H9 T cells were infected with these constructs and maintained under puromycin selection to
994 generate a chronically infected H9 T cell line expressing WT Gag (H9-HIV). In **Set IV** consists of V1B
995 constructs (see Set II), which were engineered to express WT or mutant Gag *in cis.* Set VI constructs
996 were transfected into HeLa-MCP-GFP cells, which express a GFP-tagged MS2 capsid protein (MCP) that
997 contains a nuclear localization signal. Since V1B genomic constructs also contain MS2 binding sites,
998 MCP-GFP binds to V1B, resulting in GFP tagging of V1B gRNA [8], which allows subsequent
999 immunolabeling of V1B gRNA with GFP antibody.

1000 **B) Summary of expected Gag phenotypes.** Diagram indicates, for WT Gag and Gag mutants, whether
1001 the packaging initiation complex is predicted to form and whether VLPs are known to be produced.
1002 Released VLPs that contain or lack gRNA are indicated by +(+) and +(-) respectively.

1003 **C) Confirmation of expected phenotypes for VLP production and packaging initiation complex**
1004 **formation.** COS-1 cells were transfected with Set I constructs from A. Top row: WB shows Gag in cell
1005 lysates and VLPs. Graph shows gRNA copy number, as determined by RT-qPCR, in VLPs or cell lysates
1006 representing the equivalent of 1000 cells. Similar results were obtained in 293T cells. Bottom row: Cell
1007 lysates expressing proviruses encoding WT or mutant Gag (Set I constructs in panel A) were subjected to
1008 IP with αGag or nonimmune (N) antibody followed by Gag WB (left). IP eluates were also analyzed by
1009 RT-qPCR for gRNA copies, with NI values subtracted (graph). Graph shows that only WT and G2A Gag
1010 form intracellular complexes containing Gag associated with gRNA, thereby confirming packaging

1011 initiation complex phenotypes shown in B. Error bars show SEM from duplicate samples. Data are
1012 representative of three independent repeats.
1013
1014 **Fig 2: Non-translating HIV-1 gRNA is primarily in diverse complexes >30S in the absence of**
1015 **assembling Gag**
1016 **A-E)** Lysate from COS-1 cells transfected with the assembly-incompetent MACA provirus (Fig 1A, Set
1017 I) was divided into two pools, that were either not treated or treated with PuroHS (-/+ PuroHS). Both
1018 pools were analyzed in parallel by velocity sedimentation followed by RTqPCR of paired gradient
1019 fractions using the appropriate qPCR primer sets to determine copy number of the indicated RNAs
1020 (28SrRNA, 7SL RNA, HIV-1 gRNA, HIV-1 Tat mRNA, or GAPDH mRNA). Quantity of the indicated
1021 RNAs in gradient fractions is expressed as a distribution (% of RNA in all fractions) to allow comparison
1022 between different RNAs. Blot in panel C shows migration of MACA protein, which is also graphed as a
1023 gray dotted line in C. Brackets at top show S value markers, and horizontal bars show expected
1024 migrations of various RNPCs. Error bars show SEM from duplicate samples. Data are from a single
1025 experiment that is representative of three independent repeats.
1026
1027 **Fig 3: The packaging initiation complex is an ~80S RNA granule**
1028 **A)** COS-1 cells transfected with indicated proviruses (Fig 1A, Set I) were harvested follwing PuroHS
1029 treatment, and gRNA copy number per 1000 cells was determined. **B)** Lysates from A were analyzed by
1030 velocity sedimentation, and gRNA copies per 1000 cells wasdetermined and normalized to the inputs
1031 shown in A. **C)** Gradient fractions from B were subjected to IP with HIV immune globulin (αGag), and
1032 gRNA copy number in IP eluates per 1000 cells was determined and normalized to the inputs shown in A.
1033 Similar results were obtained upon IP with monoclonal antibody to p24. **D)** COS-1 cells transfected with
1034 the indicated G2A Gag provirus (Fig 1A, Set I) were harvested following PuroHS treatment, and total
1035 gRNA copy number per 1000 cells was determined. **E)** Lysates from D were also analyzed by velocity
1036 sedimentation, and gRNA copy number per 1000 cells in each fraction was determined. **F)** Gradient

1037 fractions from E were subjected to IP with αDDX6, and IP eluates from each fraction were analyzed for
1038 gRNA copy number per 1000 cells. Brackets at top show S value markers, and dotted lines demarcate
1039 assembly intermediates based on their WB migrations. Error bars show SEM from duplicate samples.
1040 Data in each column are from a single experiment that is representative of three independent repeats.
1041
1042 **Fig 4: WT Gag forms the packaging initiation complex and a late packaging intermediate,**
1043 **including in infected human T cells**
1044 **A)** COS-1 cells transfected with the indicated construct (Fig 1A, Set II) were harvested following PuroHS
1045 treatment, and gRNA copy number per 1000 cells was determined. **B)** Lysate from A was analyzed by
1046 velocity sedimentation, and gRNA copy number per 1000 cells in each fraction was determined and
1047 normalized to the inputs shown in A. **C)** Gradient fractions from B were subjected to IP with αGFP, and
1048 gRNA copy number per 1000 cells was determined for IP eluates from each fraction and normalized to
1049 input in A. **D)** H9 cells chronically infected with the indicated provirus (H9-HIV; Fig 1A; Set III) were
1050 harvested following PuroHS treatment, and gRNA copy number per 1000 cells was determined for each
1051 fraction. **E)** Lysate from D was also analyzed by velocity sedimentation, and gRNA copy number per
1052 1000 cells in each fraction was determined. **F)** Gradient fractions from E were subjected to IP with
1053 αABCE1 and gRNA copy number per 1000 cells was determined for IP eluates from each fraction.
1054 Brackets at top show S value markers, and dotted lines demarcate assembly intermediates based on their
1055 WB migrations. Error bars show SEM from duplicate samples. Data in each column are from a single
1056 experiment that is representative of three independent repeats.
1057
1058 **Fig 5: GagZip fails to associate with gRNA-containing granules, but is rescued by a heterologous**
1059 **gRNA-binding domain**
1060 **A)** COS-1 cells transfected with indicated constructs (Fig 1A, Set II) were harvested following PuroHS
1061 treatment, and gRNA copy number per 1000 cells was determined. **B)** Lysates from A were analyzed by
1062 velocity sedimentation, and gRNA copy number per 1000 cells in each fraction was determined and

1063 normalized to the inputs shown in A. **C)** Gradient fractions from B were subjected to IP with αGFP, and
1064 gRNA copy number per 1000 cells was determined for IP eluates from each fraction and normalized to
1065 input in A. **D)** COS-1 cells transfected with indicated constructs (Fig 1A, Set II) were harvested
1066 following PuroHS treatment, and gRNA copy number per 1000 cells was determined. **E)** Lysates from D
1067 were analyzed by velocity sedimentation, and gRNA copy number per 1000 cells in each fraction was
1068 determined and normalized to the inputs shown in A. **F)** Gradient fractions from E were subjected to IP
1069 with αGFP, and gRNA copy number per 1000 cells was determined for IP eluates from each fraction.
1070 Brackets at top show S value markers, and dotted lines demarcate assembly intermediates based on their
1071 WB migrations. Error bars show SEM from duplicate samples. Data in each column are from a single
1072 experiment that is representative of two independent repeats.
1073

1074 **Fig 6: Gag-DDX6 colocalization *in situ* upon provirus expression**

1075 PLA was used to detect regions in which Gag is within 40 nm from DDX6 *in situ*, with concurrent αGag
1076 IF for quantification of intracellular Gag levels. **A)** Experimental schematic and diagrams of proviruses
1077 transfected into 293T cells (Fig 1A, Set 1). **B)** The average number of PLA spots per cell was determined
1078 for all Gag-positive cells in five randomly chosen fields, and normalized to Gag levels. Error bars show
1079 SEM. **C)** Representative images. From left to right for each construct: Gag IF (green) with DAPI-stained
1080 nuclei (blue), Gag-DDX6 PLA signal (red) with DAPI-stained nuclei (blue), and a merge of all three.
1081 Merge demonstrates that PLA spots are mainly in Gag-expressing cells. Inset in PLA panel shows a high
1082 magnification view of the cell to the right of the inset. Scale bars, 5 μM. Data are representative of three
1083 independent repeats. Error bars show SEM (n=10 cells). ** indicates a significant difference relative to
1084 WT ($p \leq 0.001$).
1085

1086 **Fig 7: Gag-ABCE1 colocalization *in situ* upon provirus expression**

1087 PLA was used to detect regions in which Gag is within 40 nm from ABCE1 *in situ*, with concurrent αGag
1088 IF for quantification of intracellular Gag levels. **A)** Experimental schematic and diagrams of proviruses
1089 transfected into 293T cells (Fig 1A, Set 1). **B)** The average number of PLA spots per cell was determined
1090 for all Gag-positive cells in five randomly chosen fields, and normalized to Gag levels. Error bars show
1091 SEM. **C)** Representative images. From left to right for each construct: Gag IF (green) with DAPI-stained
1092 nuclei (blue), Gag-ABCE1 PLA signal (red) with DAPI-stained nuclei (blue), and a merge of all three.
1093 Merge demonstrates that PLA spots are mainly in Gag-expressing cells. Inset in PLA panel shows a high
1094 magnification view of the cell to the left of the inset. Scale bars, 5 μM. Data are representative of three
1095 independent repeats. Error bars show SEM (n=10 cells). * indicates a significant difference relative to
1096 WT ($p \leq 0.01$).
1097
1098 **Fig 8: Packaging initiation complexes containing Gag and DDX6 are far more numerous than P**
1099 **bodies**
1100 PLA was used to identify regions in which Gag is within 40 nm from DDX6 *in situ*, with concurrent
1101 αDDX6 IF for quantification of P bodies. **A)** Experimental schematic. 293T cells were transfected with
1102 the indicated G2A provirus (Fig 1A, Set 1) or mock transfected. **B)** The average number of P bodies per
1103 cell (green bar) or PLA spots per cell (red bar) was determined for all Gag-positive cells in five randomly
1104 chosen fields. Error bars show SEM (n=10 cells). **C)** Representative images**.** From left to right for G2A
1105 Gag and Mock: DDX6 IF to detect P bodies (green) overlaid with nuclei (blue), PLA signal (red)
1106 overlaid with nuclei (blue), and a merge of all three. Insets in DDX6 IF panels show a high gain/high
1107 magnification version of cell to left, revealing a diffuse, low intensity DDX6 signal (example in dotted
1108 oval). Arrows indicate the same two P bodies in low and high magnification images. Insets in merged
1109 panels show a high gain/high magnification version of two cells to right. Scale bar, 5 μM. Data are
1110 representative of three independent repeats.
1111

1112 **Fig 9. The gRNA-DDX6 association at PM assembly sites is observed *in situ* for WT Gag but not for**
1113 **GagZip**

1114 **A)** HeLa cells expressing MCP-GFP were transfected with V1B genomes that contain MS2 binding sites
1115 (Fig 1A, Set IV constructs) and express WT Gag (shown), GagZip, or MACA (diagrams not shown).
1116 Cells were analyzed by double-label IEM, using αDDX6 (large gold), and αGFP, which allowed detection
1117 of MCP-GFP labeled gRNA (small gold). All early and late assembly sites at the PM were identified in
1118 ten cells (~250 μm of PM total per group), and scored for gRNA labeling, DDX6 labeling, and double
1119 labeling. **B)** Graph shows the percentage of all early and late PM assembly events that are DDX6-labeled
1120 (total DDX6+), gRNA-labeled (total gRNA +), or double-labeled (gRNA+/DDX6+). Error bars show
1121 SEM (n=10 cells). *** indicates a significant difference between WT Gag and GagZip ($p<0.005$). For
1122 additional data, see Table S1. **C)** Images show representative assembly sites at the PM for each group,
1123 with symbols indicating early or late PM assembly sites that are single-labeled for either gRNA or DDX6
1124 (dark arrows), double-labeled (asterisks), or unlabeled (open arrows). Dotted lines outline the PM. Scale
1125 bars, 200 nm.

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1127 **Fig 10. Model for how HIV-1 gRNA packaging is initiated within a subclass of host RNA granules**

1128 **A)** MACA Gag, which lacks NC, fails to associate with RNA granules. In contrast, the targeting-
1129 defective G2A Gag mutant associates with ~80S RNA granules that contain gRNA, leading to packaging
1130 initiation complex formation in the cytoplasm without further progression. WT Gag also associates with
1131 RNA granules and forms the packaging initiation complex in the cytoplasm, which is then targeted to the
1132 PM where Gag multimerizes to complete assembly. RNA granule proteins dissociate from the fully
1133 assembled capsid prior to VLP budding and release. GagZip also targets to ~80S RNA granules, but
1134 because it contains a protein-protein dimerization domain in place of the gRNA-binding NC domain it is
1135 unable to target to the subset of ~80S granules that contains gRNA. Ultimately, GagZip also undergoes
1136 PM targeting, assembly, dissociation of RNA granule proteins, and VLP release; however, GagZip VLPs
1137 lack gRNA because the gRNA-binding-deficient GagZip targeted to the "wrong" subset of ~80S granules.

1138 **B)** The two-step model for initial targeting of Gag to gRNA-containing RNa granules is shown in more
1139 detail. Only a subset of ~80S RNA granules contain gRNA. While MACA Gag does not associate with
1140 any RNA granule subsets, G2A and WT Gag target to the subset of RNA granules that contains gRNA.
1141 GagZip targets to RNA granules regardless of whether they contain gRNA; however, a heterologous
1142 gRNA-binding domain (MCP) is able to rescue GagZip, allowing it to target to gRNA-containing ~80S
1143 granules.
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1164 **Supporting Information Captions:**

1165 **Fig. S1: Packaging initiation and VLP phenotypes for V1B *in trans* and *in cis* constructs.**

1166 **A)** COS-1 cells were cotransfected with WT or mutant codon-optimized Gag fused to GFP (GagGFP)
1167 along with a genomic construct (V1B) provided *in trans* (Fig. 1A, Set II). Diagram shows schematic of
1168 constructs. Top: Gag WB of cells and VLPs. Graph shows gRNA copy number in VLP pellets from the
1169 equivalent of 1000 cells, as determined by RT-qPCR. Bottom: Lysates of cells transfected as in A were
1170 subjected to IP with αGFP or nonimmune (N) antibody followed by Gag WB (left), with IP inputs shown
1171 (center).

1172 **B)** HeLa cells expressing MCP-GFP were transfected with V1B genomes that contain MS2 binding sites
1173 and express WT Gag, G2A, GagZip, or MACA (Fig. 1A, Set IV constructs). Diagram shows schematic
1174 of constructs. Also shown is a Gag WB of cells and VLPs. Graph shows gRNA copy number in VLP
1175 pellets from the equivalent of 1000 cells, as determined by RT-qPCR, with mock background for VLPs
1176 subtracted.

1177

1178 **Fig. S2: Association of gRNA with Gag and ABCE1 in the ~80S packaging initiation complex and**
1179 **~500S late packaging intermediate.**

1180 **A)** 293T cells transfected with the indicated WT Gag construct (Fig. 1A, Set II) were harvested +
1181 PuroHS, and gRNA copy number per 1000 cells was determined. **B)** Lysates from B were also analyzed
1182 by velocity sedimentation, and gRNA copy number from the equivalent of 1000 cells determined. **C)**
1183 Gradient fractions from B were subjected to IP with αGFP, and gRNA copy number in IP eluates per
1184 1000 cells determined. **D)** COS-1 cells transfected with the indicated construct (Fig. 1A, Set II) were
1185 harvested and analyzed as in A. **E)** Lysate from D was analyzed as in B. **C)** Gradient fractions from E
1186 were subjected to IP with αABCE1, and gRNA copy number in IP eluates from the equivalent of 1000
1187 cells determined. Brackets at top show S value markers, and dotted lines demarcate assembly
1188 intermediates based on their migrations in the Gag WB. Error bars, SEM from duplicate samples. Data
1189 represent three independent repeats.

1190 **Fig. S3: Immunoprecipitation and VLP analysis of GagZip constructs**

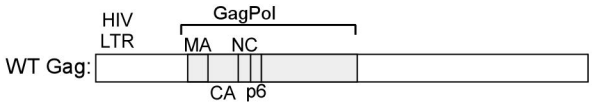
1191 **A)** COS-1 cells were cotransfected with a codon-optimized construct expressing either WT Gag or
1192 GagZip fused to GFP and a genomic construct (V1B) provided *in trans*. Cells were harvested following
1193 PuroHS treatment and analyzed by velocity sedimentation. Paired gradient fractions were subjected to
1194 αGFP IP, followed by WB with HIV immune globulin to allow detection of Gag. **B)** COS-1 cells
1195 transfected with indicated constructs (Fig. 1A, Set II) and lysates and VLPs were harvested. WB shows
1196 Gag in cell lysates and VLPs. Graph shows gRNA copy number, as determined by RT-qPCR, in VLPs or
1197 cell lysates representing the equivalent of 1000 cells.

1198

Fig. 1: Gag constructs: diagrams and phenotypes

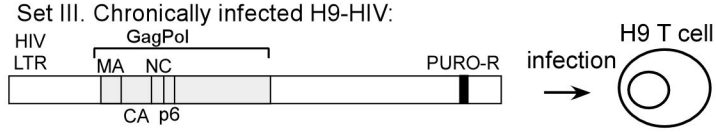
A

Set I. Transfected HIV-1 provirus (mutants not shown):

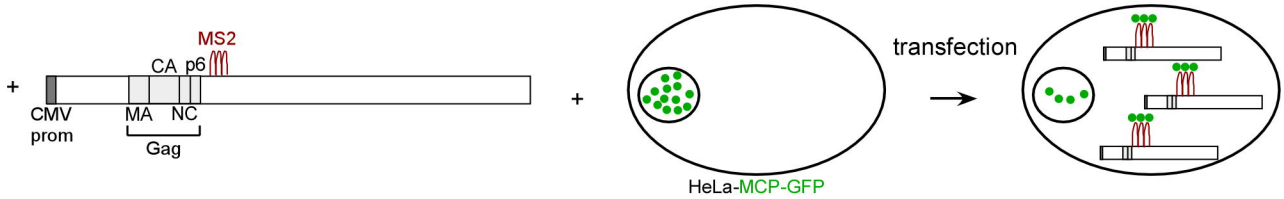


Set II. Transfected GagGFP +/- V1B-MS2 genome *in trans* (mutants not shown):

Set III. Chronically infected H9-HIV:



Set IV. V1B-MS2 genome expressing Gag *in cis* transfected into HeLa-MCP-GFP (mutants not shown):



B

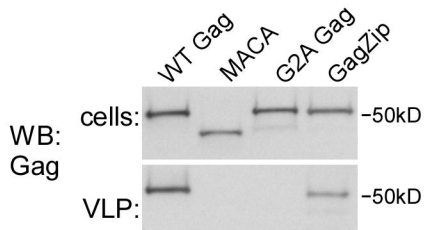
Gag phenotypes:

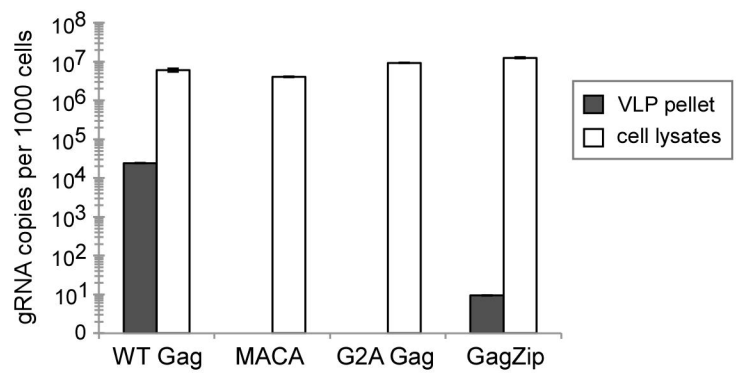
	Gag (proviral)	GagGFP *in trans*	gRNA packaging initiation complex:	VLP (+/- gRNA):
WT Gag:	MA CA NC p6	MA CA NC p6	+	+ (+)
MACA:	MA CA	MA CA	-	-
G2A Gag:	* MA CA NC p6	* MA CA NC p6	+	-
Gag Zip:	MA CA Zip p6	MA CA Zip p6	-	+ (-)

C

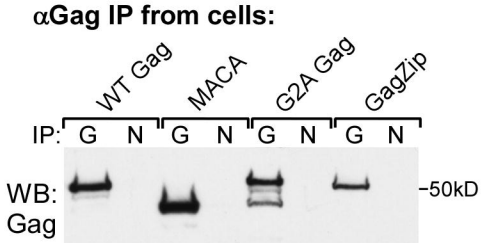
HIV-1 provirus (Set I):

VLP:





αGag IP from cells:



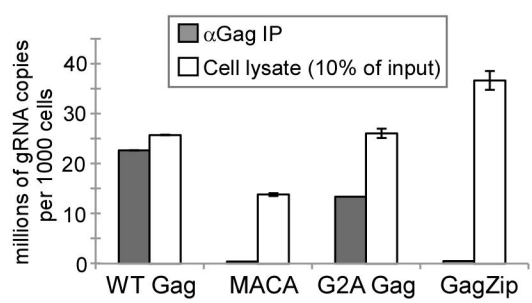


Fig. 2: Non-translating HIV-1 gRNA is primarily in diverse complexes >30S in the absence of assembling Gag

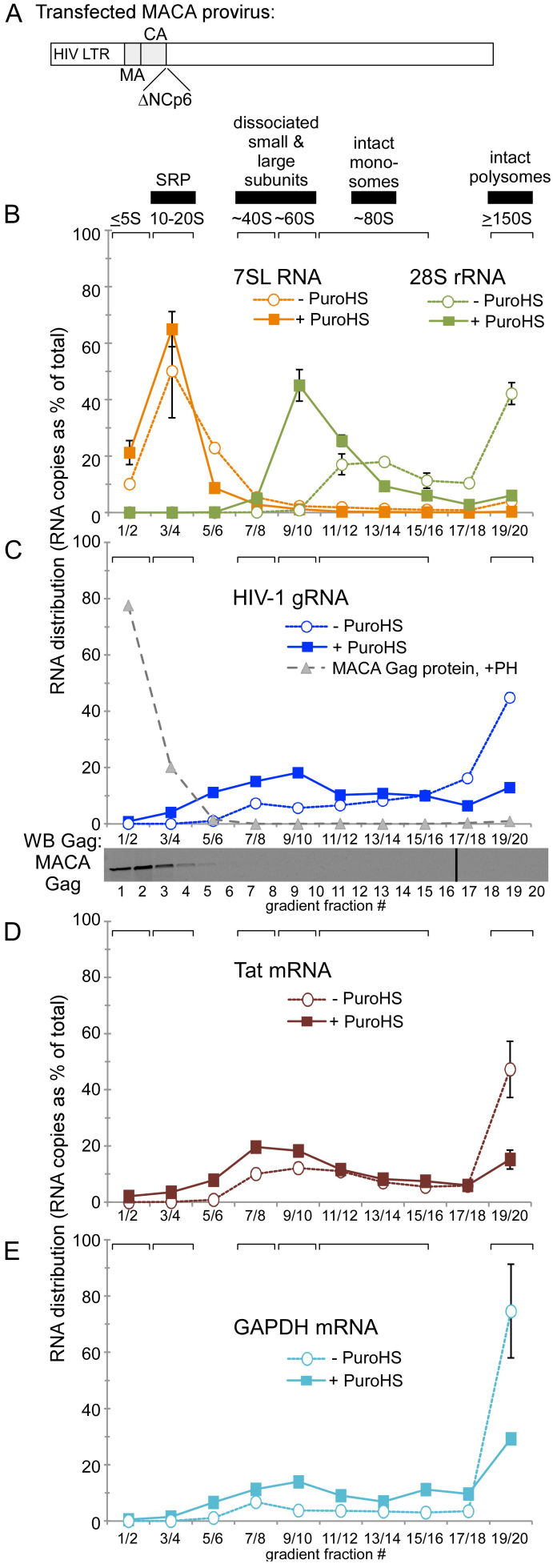


Fig. 3: The packaging initiation complex is an ~80S RNA granule

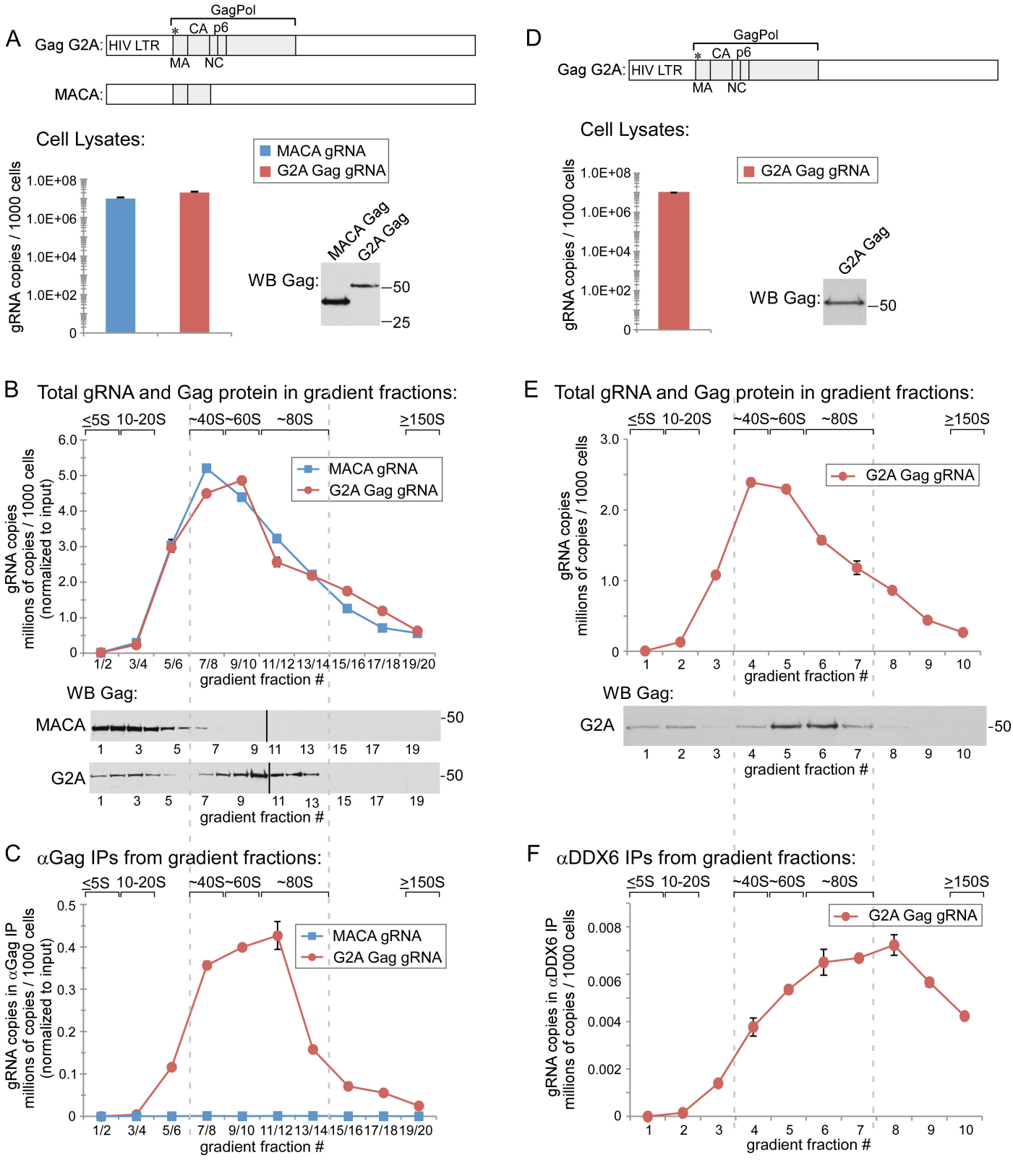


Fig. 4: WT Gag forms the packaging initiation complex and a late packaging intermediate, including in infected human T cells

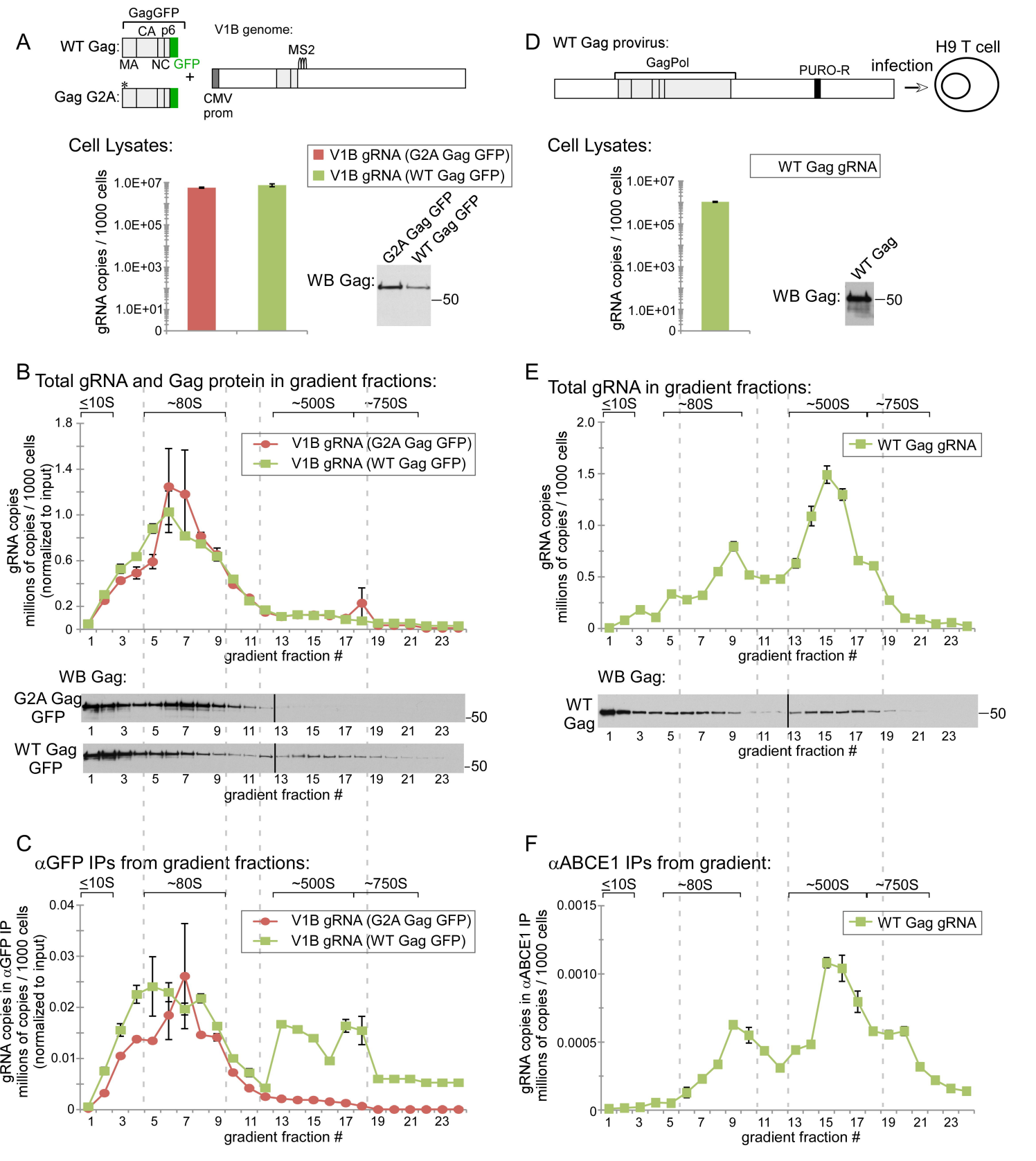


Fig. 5: GagZip fails to associate with gRNA-containing granules, but is rescued by a heterologous gRNA binding domain

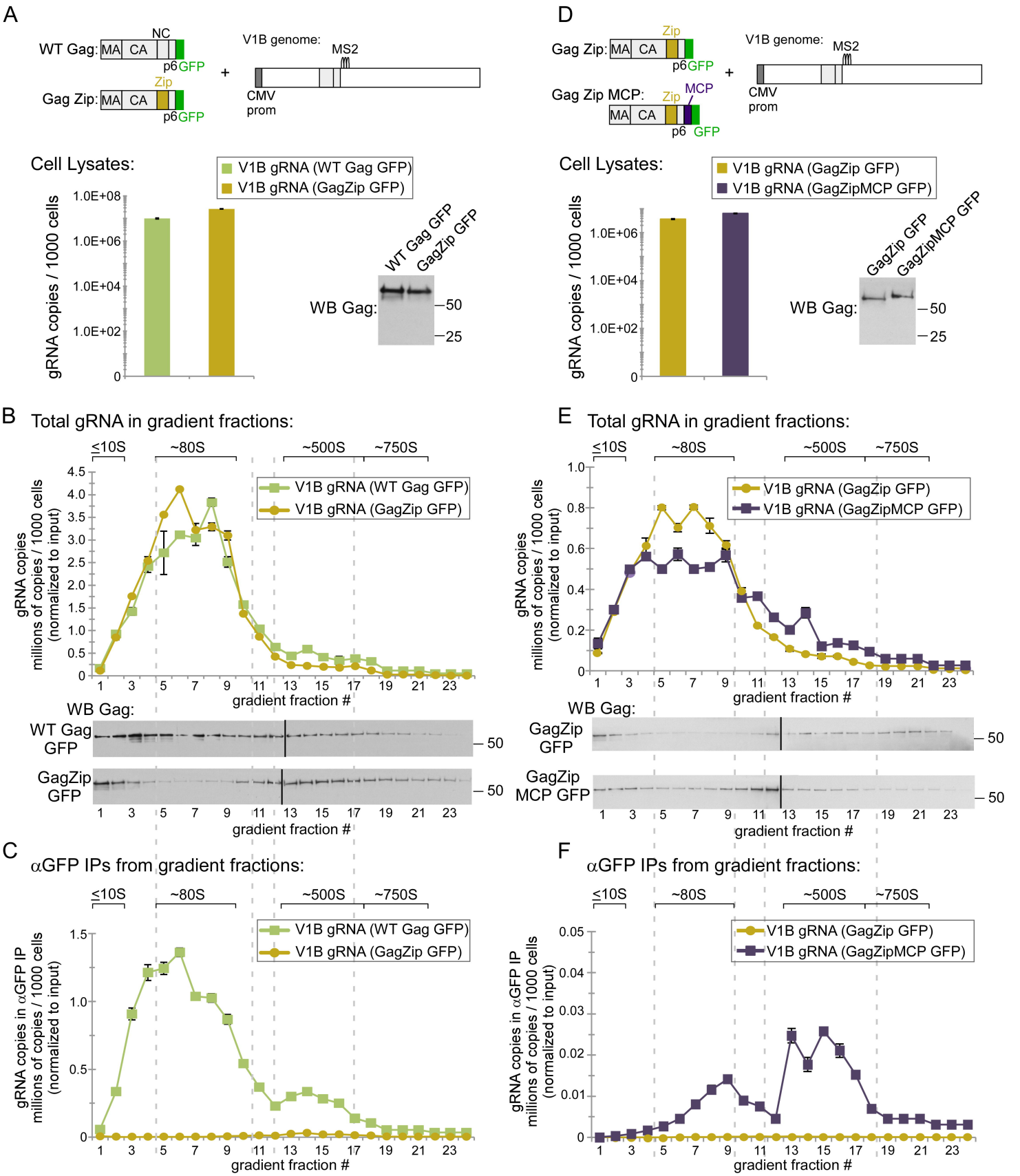
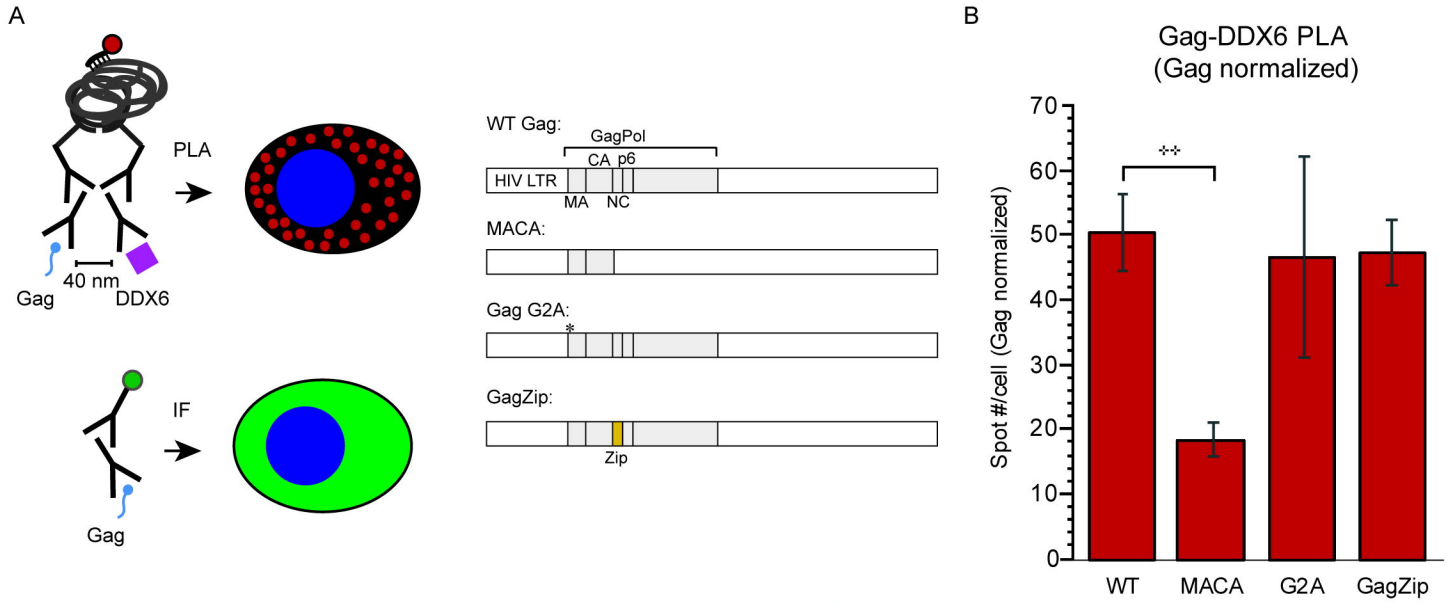


Fig. 6: Gag-DDX6 colocalization *in situ* upon provirus expression



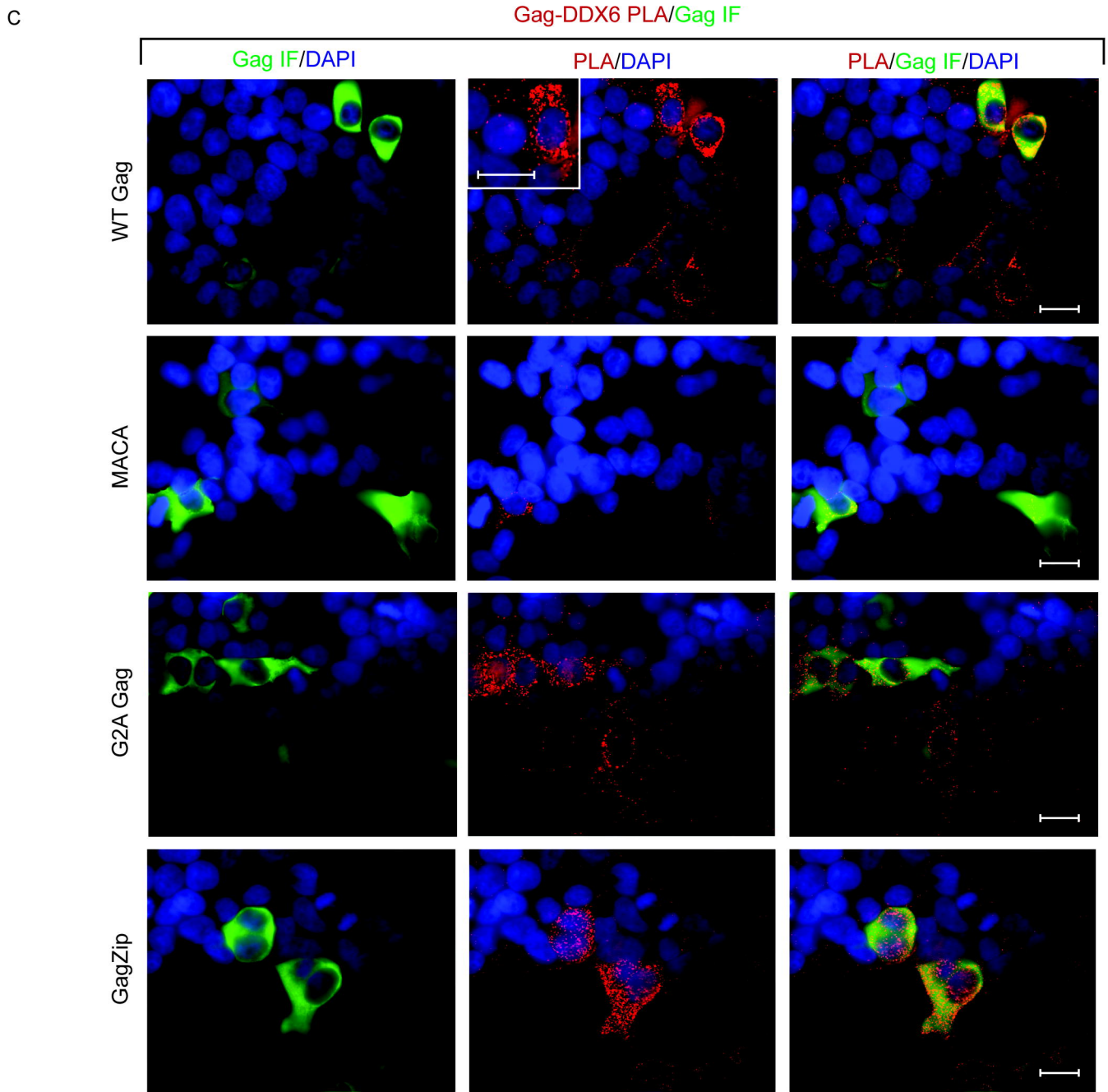
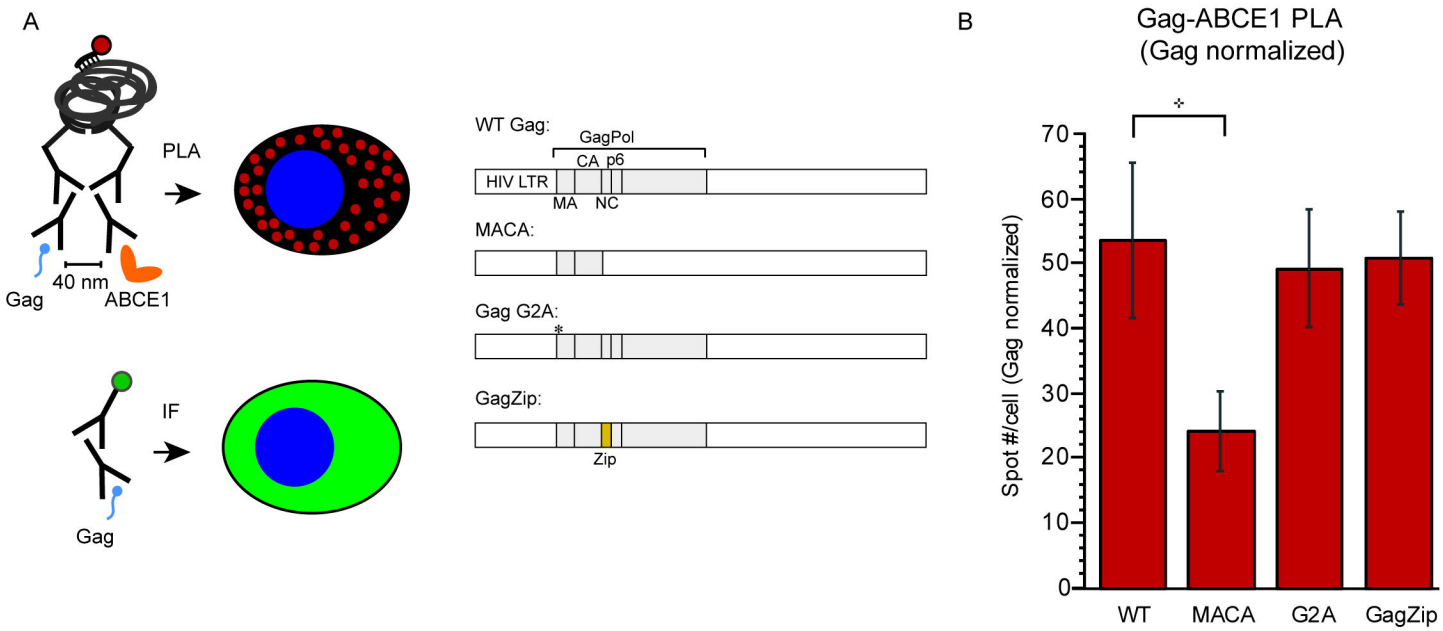


Fig. 7: Gag-ABCE1 colocalization *in situ* upon provirus expression



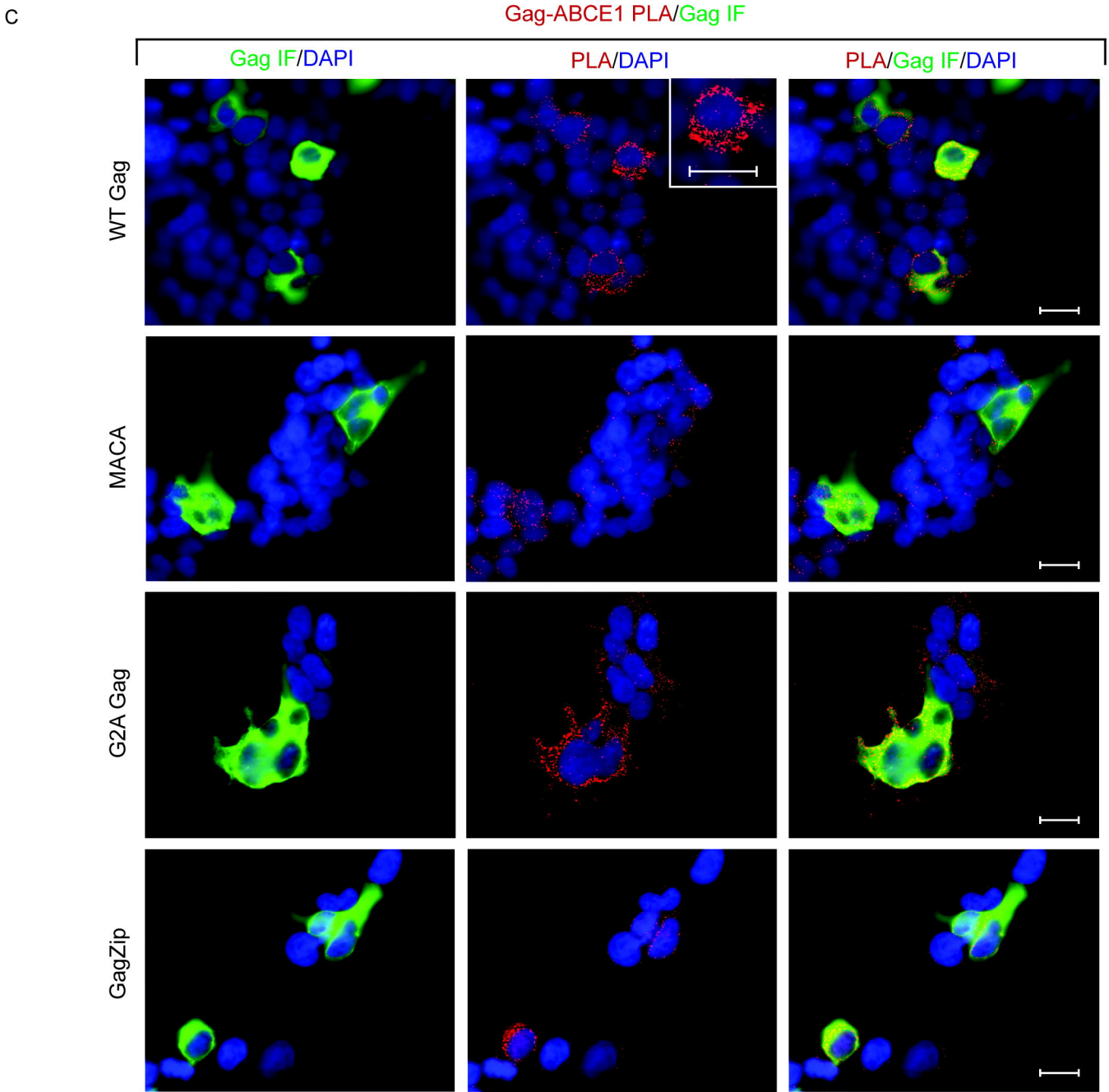
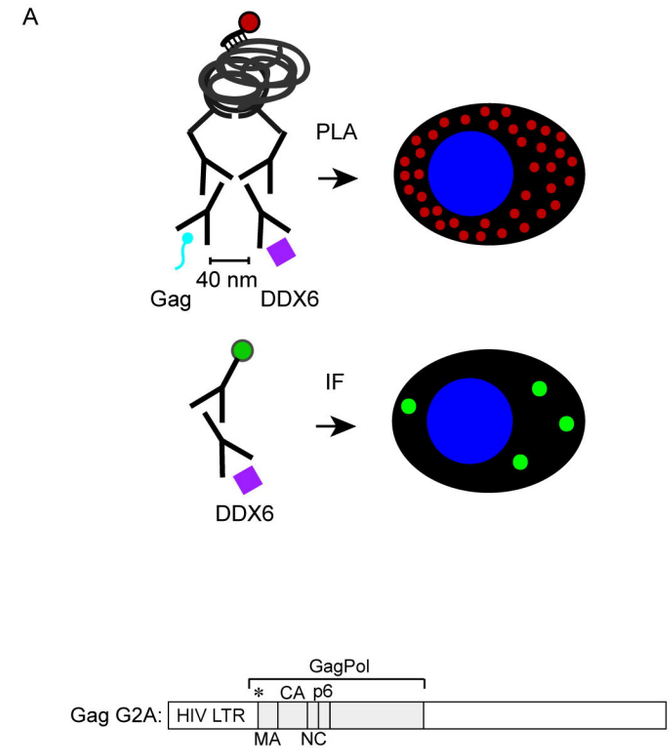
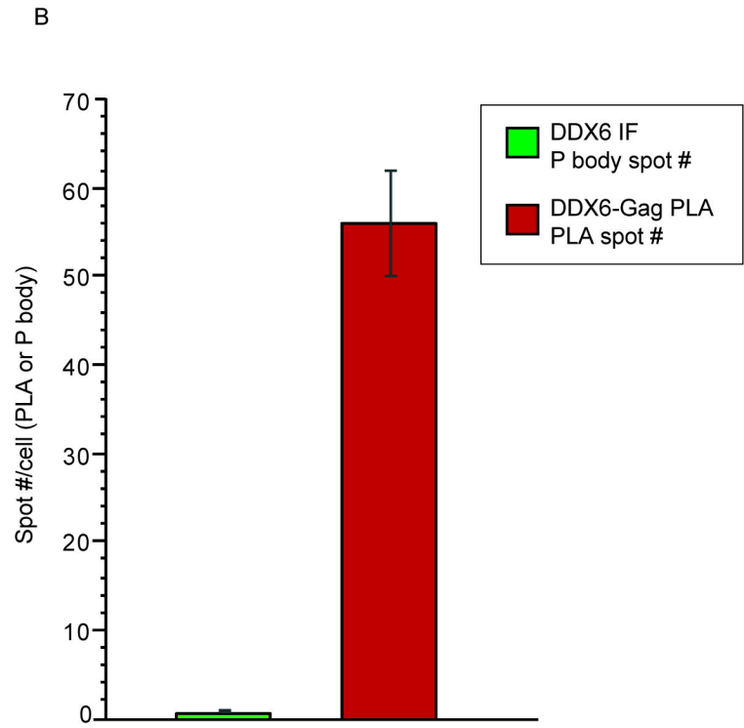


Fig. 8: Packaging initiation complexes containing Gag and DDX6 are far more numerous than P bodies.





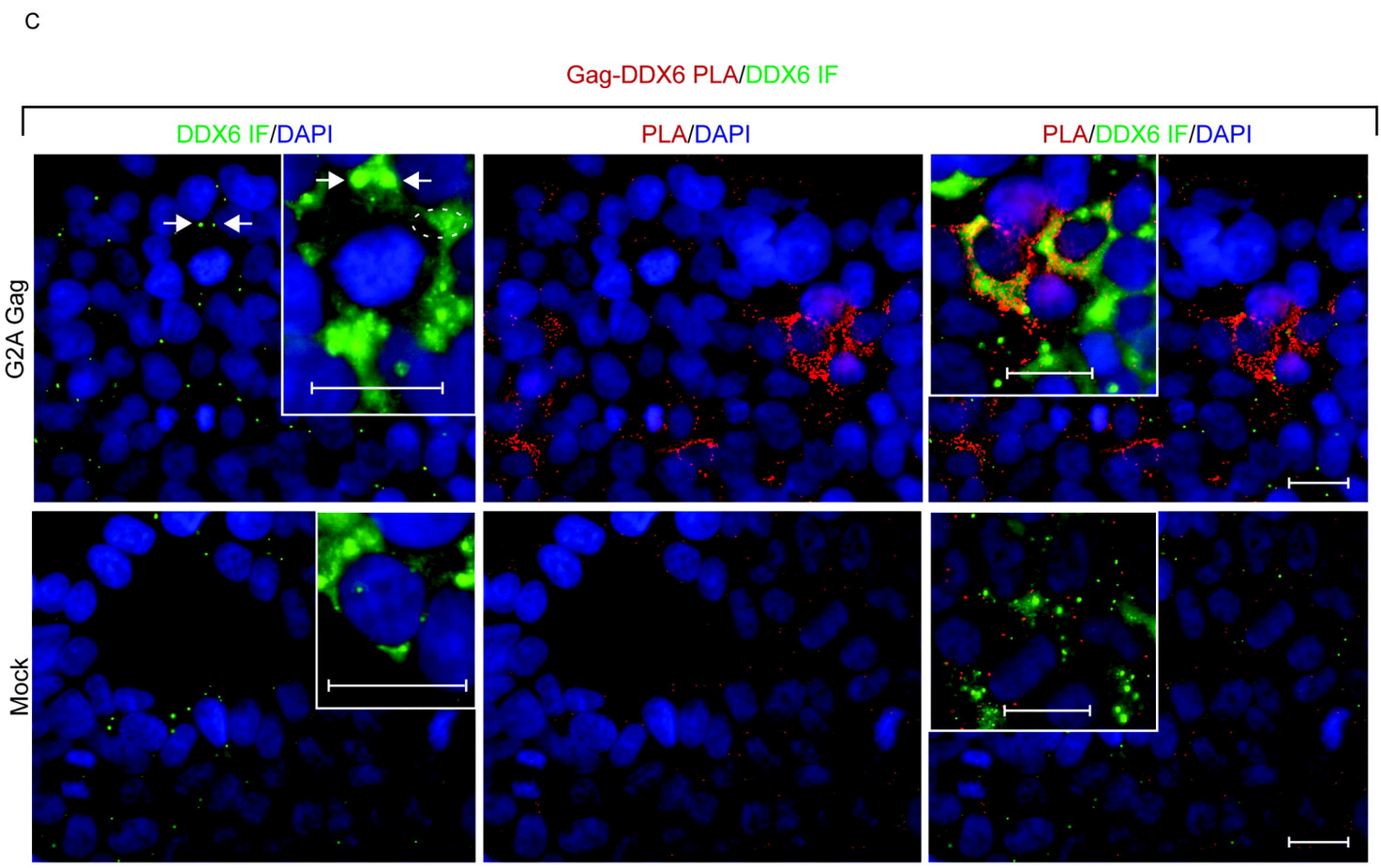
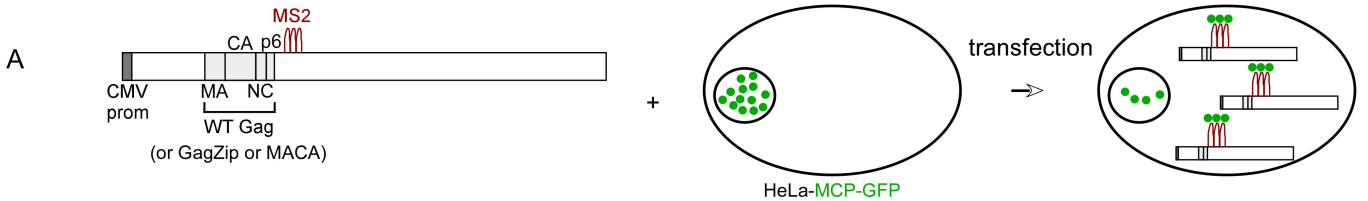
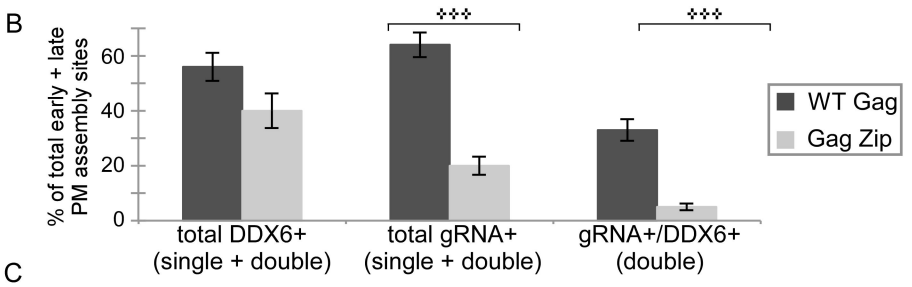


Fig. 9. The gRNA-DDX6 association at PM assembly sites is observed *in situ* for WT Gag but not for GagZip





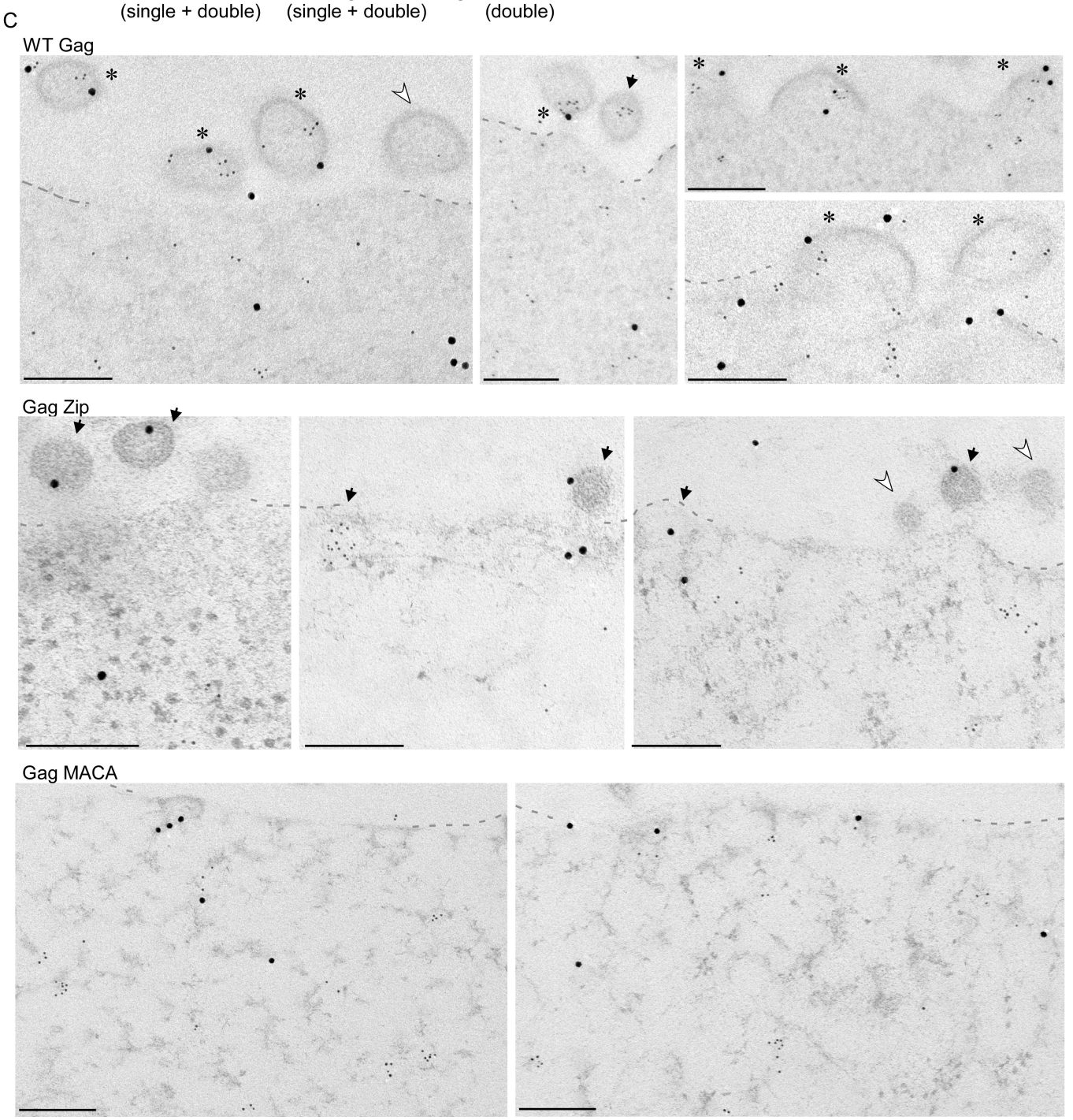
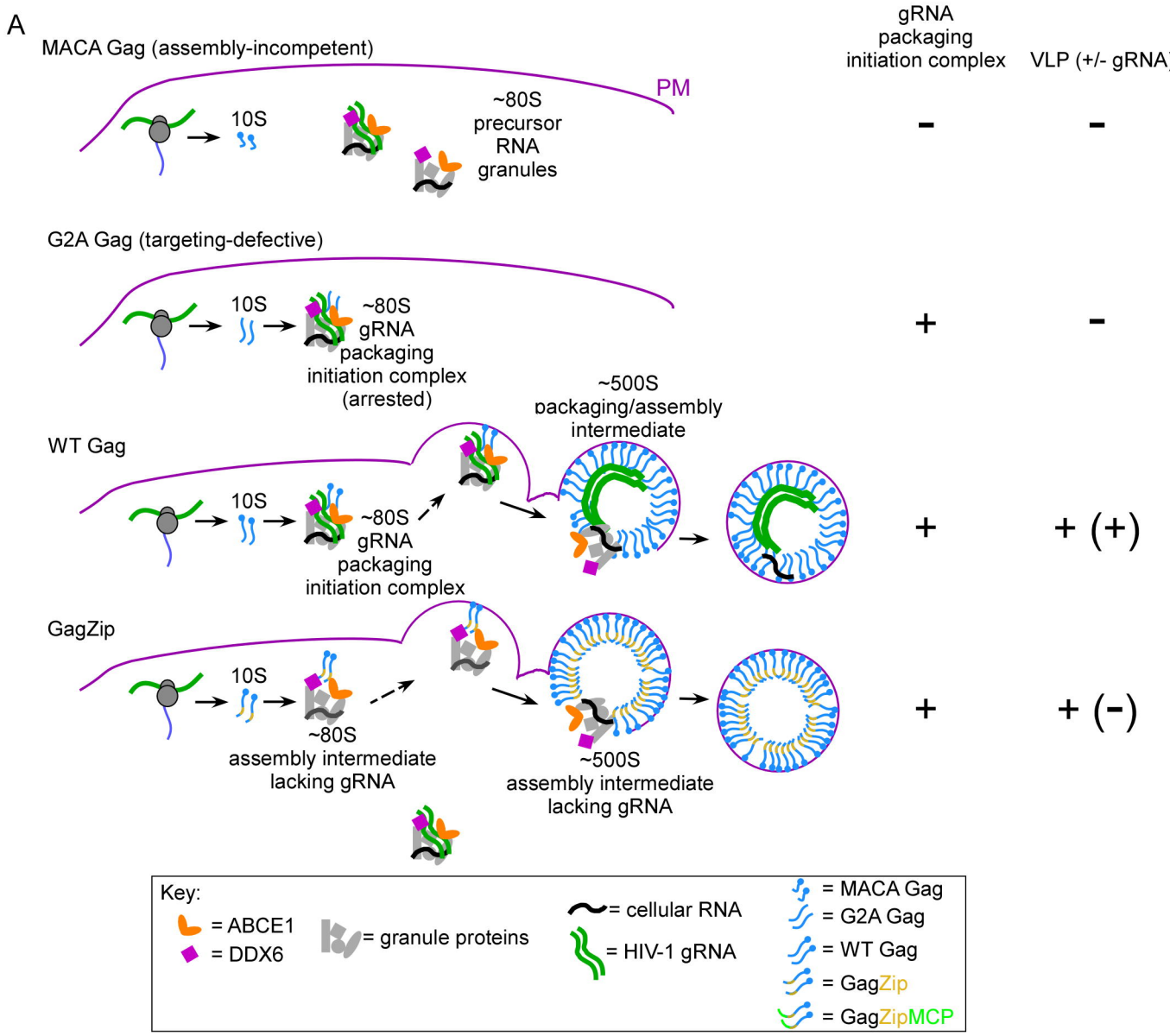
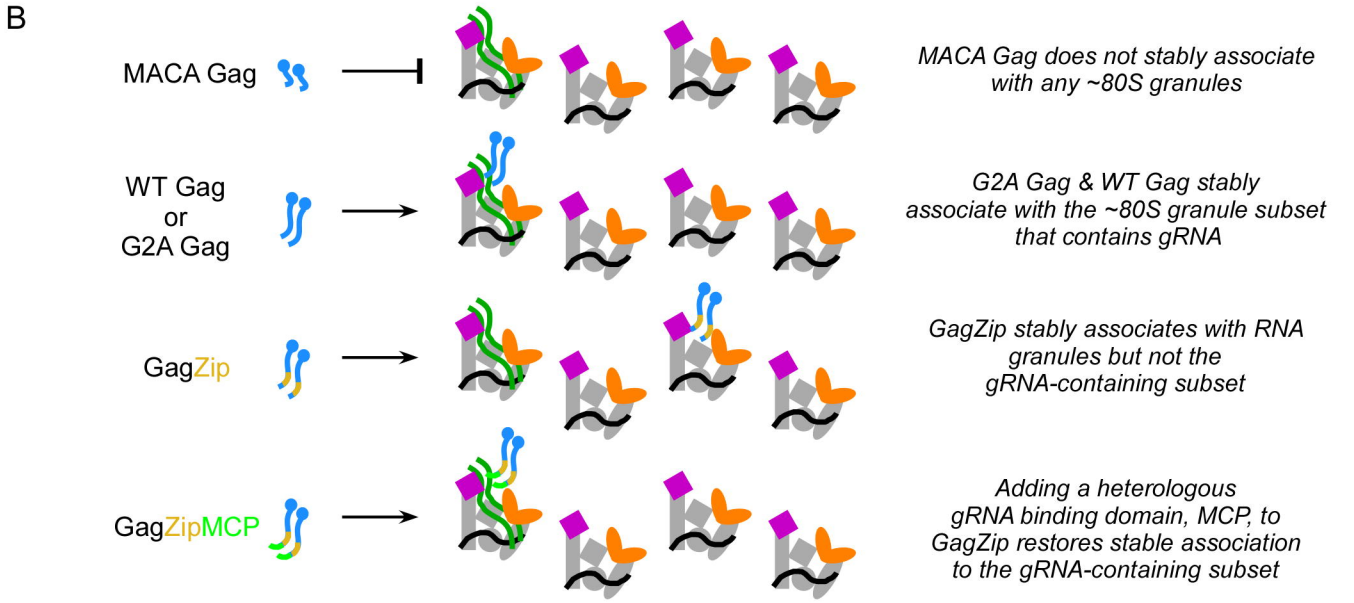



Fig. 10: Model for how HIV-1 gRNA packaging is initiated within a subclass of host RNA granules