Full assembly of HIV-1 particles requires assistance of the membrane curvature factor IRSp53.

Authors

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26 **Abstract**

27 During HIV-1 particle formation, the requisite plasma membrane curvature is thought to be solely
28 driven by the retroviral Gag protein. Here, we reveal that the cellular I-BAR protein IRSp53 is
29 required for the progression of HIV-1 membrane curvature to complete particle assembly.
30 SiRNA-mediated knockdown of IRSp53 gene expression induces a decrease in viral particle
31 production and a viral bud arrest at half completion. Single molecule localization microscopy at
32 the cell plasma membrane shows a preferential localization of IRSp53 around HIV-1 Gag
33 assembly sites. In addition, we observe the presence of IRSp53 in purified HIV-1 particles.
34 Finally, HIV-1 Gag protein preferentially localizes to curved membranes induced by IRSp53 I-
35 BAR domain on giant unilamellar vesicles. Overall, our data reveal a strong interplay between
36 IRSp53 I-BAR and Gag at membranes during virus assembly. This highlights IRSp53 as a crucial
37 host factor in HIV-1 membrane curvature and its requirement for full HIV-1 particle assembly.

38

39 **Introduction**

40 The cell plasma membrane is a dynamic structure, where crucial processes such as
41 endocytosis and exocytosis take place through local membrane deformations. Several pathogens,
42 such as bacteria and enveloped viruses interplay with the plasma membrane in the course of their
43 replication cycle. Pathogens often enter the cells by endocytosis (Grove and Marsh, 2011;
44 Gruenberg and van der Goot, 2006) and exit by membrane vesiculation (Rheinemann and
45 Sundquist, 2020; Welsch et al., 2007), which are processes linked to the generation of plasma
46 membrane curvature; either inward or outward. HIV-1 is an enveloped positive strand RNA virus
47 belonging to the family *Retroviridae*, and it is known to assemble and bud outward from the host
48 cell plasma membrane (Coffin et al., 1997). The structural Gag polyprotein of HIV-1, by itself, is
49 responsible for particle assembly (Gheysen et al., 1989); it can oligomerize at the inner leaflet of
50 the plasma membrane forming virus-like particles (VLPs). The force required to bend the
51 membrane to achieve VLP formation has been proposed to be provided by Gag self-assembly

(Hurley et al., 2010). The self-assembly of Gag has also been recently shown to segregate specific lipids (Favard et al., 2019; Yandrapalli et al., 2016) and proteins (Sengupta et al., 2019), generating plasma membrane domains that could favor budding (Foret, 2014; Lipowsky, 1993). However, only a small proportion of Gag-initiated clusters reach the fully assembled state leading to VLP release in living CD4 T cells (Floderer et al., 2018), while a majority of these clusters lead to aborted events. Therefore, the mechanism by which the virus overcomes the energy barrier associated with the formation of the full viral bud remains an open question. Recently, a coarse grained model of HIV assembly has shown that the self-assembly of Gag might not be sufficient to overcome this energy barrier (Pak et al., 2017), leaving the assembly in intermediate states. This supports the fact that other factors may be necessary to assist Gag self-assembly during the generation of new VLPs.

Plasma membrane curvature can also be generated by diverse host cell proteins. For example, I-BAR domain proteins sense and induce negative membrane curvature at the nanometer scale (a few tens to one hundred nanometers), of i.e., in the HIV-1 particle diameter size range, while generating outward micrometer scale membrane protrusions such as membrane ruffles, lamellipodia, and filopodia. IRSp53 was first discovered as a substrate phosphorylated downstream of the insulin receptor (Yeh et al., 1996). It is also the founding member of the membrane curving I-BAR domain protein family, whose other mammalian members are MIM (missing-in-metastasis), ABBA (actin-bundling protein with BAIAP2 homology), PinkBAR (planar intestinal and kidney specific BAR domain protein), and IRTKS (insulin receptor tyrosine kinase substrate) (Zhao et al., 2011). The latter, IRTKS, displays functional redundancy with IRSp53 (Chou et al., 2017; Millard et al., 2007) in being able to curve membranes. In addition to interactions with the plasma membrane, IRSp53 binds both Rac1, through its N-terminal I-BAR domain (Miki et al., 2000) and Cdc42 directly through its unconventional CRIB domain (Krugmann et al., 2001); downstream effectors of these GTPases such as WAVE2, Mena, Eps8

77 and mDia can bind IRSp53 through the SH3 domain. Thus, IRSp53 functions as a scaffold protein
78 for the Rac1/Cdc42 cascade (Scita et al., 2008). IRSp53 was reported to exhibit a closed inactive
79 conformation that opens synergistically upon binding to Rac1/Cdc42 and effector proteins
80 (Disanza et al., 2013; Kast and Dominguez, 2019; Miki and Takenawa, 2002; Suetsugu et al.,
81 2006). Regulation of IRSp53 activity was recently shown to occur through its phosphorylation
82 and interaction with 14-3-3(Kast and Dominguez, 2019). Structurally, the I-BAR domain of
83 IRSp53 is composed of a rigid six alpha-helix bundle dimer that is *crescent*-shaped. Due to its
84 concave membrane binding surface and lipid interactions, IRSp53 is able to generate negative
85 membrane curvature (Zhao et al., 2011). While capable of forming homo-dimers, IRSp53 is also
86 able to recruit and form hetero-dimers with other proteins to form clusters for the initiation of
87 membrane curvature (Disanza et al., 2013).

88 Since the Rac1/IRSp53/Wave2/Arp2/3 signaling pathway is involved in the release of
89 HIV-1 particles (Thomas et al., 2015), we hypothesized that IRSp53 may be a prime candidate for
90 membrane remodeling required during viral bud formation. Hence, we investigated the possible
91 role of IRSp53 and its membrane curvature generating activity in HIV-1 Gag assembly and
92 particle budding. Importantly, we discovered that IRSp53 is present in an intracellular complex
93 with HIV-1 Gag at the cell membrane, incorporated in Gag-VLPs and it is associated with
94 purified HIV-1 particles, supporting IRSp53's function in HIV-1 assembly as a facilitator of
95 optimal HIV-1 particle formation through its membrane bending activity. Thus, we identify
96 IRSp53 as an essential non-redundant novel factor in HIV-1 replication, and demonstrate that it is
97 critical for efficient HIV-1 membrane curvature and full assembly at the cell plasma membrane.

98 **Results**

99 **1. IRSp53 knockdown decreases HIV-1 Gag particle release by arresting its assembly at**
100 **the cell plasma membrane.**

101 We report here that the partial knockdown of IRSp53 expression reduces HIV-1 particle
102 release in host Jurkat T cells and in the model cell line HEK293T (Fig 1a, b), similar to our
103 previously reported results in primary T lymphocytes (Thomas et al., 2015). Cells were treated
104 with siRNA targeting IRSp53 or IRTKS (validated by extinction of the transfected ectopic
105 IRSp53-GFP or IRTKS-GFP proteins – Fig 1-Fig S1b and S1c, respectively). In Jurkat T cells,
106 we expressed the viral Gag proteins in the context of HIV-1(Δ Env) in order to only monitor the
107 late steps of the viral life cycle. Partial IRSp53 gene expression knockdown (resulting in a
108 maximum of 50% protein depletion) reduced particle release 3-fold as compared to the control
109 siRNA (Fig 1a, bottom), and a 6-fold reduction was determined when taking into account the
110 percentage of protein depletion. This reduction in HIV-1 particle release is highly significant (n=3
111 independent experiments, p value = 0.00265, Student's *t*-test, and ANOVA statistical test for
112 multiple comparison indicates p value = 0.0089) since the gene editing of IRSp53 cannot be
113 complete, nor edited by CRISPR/Cas9 knockout, without being toxic for the cells. To compare
114 the role of different I-BAR domain proteins from the same family, we also measured the effect of
115 siRNA targeting IRSp53 and IRTKS (Fig 1b) on HIV-1 Gag virus-like particle (VLP) production
116 in HEK293T cells (Fig 1-S1a, see graph Fig 1b, and immunoblots Fig 1-Fig S1d, e). IRTKS
117 shares similar protein domain organization and high sequence homology with IRSp53 (40%
118 amino acid sequence identity and 59% sequence similarity, Fig 1-Fig S2), and displays some
119 functional redundancy with IRSp53 (Chou et al., 2017). IRTKS can also induce plasma
120 membrane curvature (Saarikangas et al., 2009). Partial knockdown of IRSp53 (~50% protein
121 depletion) resulted in a 2- to 3-fold decrease in HIV-1 Gag particle production (n=3 independent
122 experiments, p value = 0.000487, Student's *t*-test) (Fig 1b, bottom, S1d). In contrast, knockdown

123 of IRTKS (Fig 1b, S1e) did not have any significant effect on HIV Gag particle release (n=3
124 independent experiments, p value = 0.0924, Student's *t*-test), thus precluding the possibility of
125 redundant functions between IRSp53 and IRTKS in the context of HIV-1 Gag particle formation.

126 Electron microscopy imaging of siRNA IRSp53 treated HEK293T cells expressing HIV-1
127 Gag revealed arrested particle budding at the cell plasma membrane (Fig 1c, Lower Panel), as
128 compared to the siRNA-control cells (Fig 1c, Upper Panel). While the control cells exhibited the
129 normal phenotype of Gag-VLP budding from the cell plasma membrane, the IRSp53 knockdown
130 cells displayed a series of viral buds arrested in assembly decorating the cell plasma membrane
131 (Fig 1c, Fig 1-Fig S3). These results revealed an arrest in Gag assembly at the membrane and thus
132 the involvement of IRSp53 in the assembly process. Since IRSp53 is an I-BAR protein involved
133 in cell membrane curvature, we measured the curvature exhibited by HIV-1 buds in IRSp53
134 knockdown cells. While control cells displayed a range of HIV-1 Gag particles at different stages
135 of assembly and budding, cells knocked down for IRSp53 instead displayed arrested buds at an
136 early assembly stage (Fig 1d). By measuring the dimensions of these arrested buds, we found that
137 buds from cells knocked down for IRSp53 displayed a narrower range of curvature height ($48 \pm$
138 22nm) as compared to the control ($85 \pm 53\text{ nm}$) (n=145 buds from 14 different cells , p value =
139 1.053×10^{-28} Kolmogorov-Smirnov test), while the bud widths presented no difference between
140 siIRSp53 ($135 \pm 64\text{nm}$) and the control ($140 \pm 87\text{ nm}$)) (n=145 buds from 14 different cells, p
141 value = 0.0609, Kolmogorov-Smirnov test) (Fig 1e). The control cells thus exhibited a range of
142 height and widths consistent with the range of buds seen at the membrane of these cells. The
143 result indicates that in the absence of IRSp53, the viral buds were unable to progress beyond a
144 certain curvature.

145

146 **2. HIV-1 Gag expression in cells increases IRSp53 membrane binding and allows their**
147 **complexation.**

148 Since both Gag and IRSp53 target the cell plasma membrane upon interaction with PI(4,5)P₂
149 (Favard et al., 2019; Mattila et al., 2007; Prévost et al., 2015; Saarikangas et al., 2009; Sengupta
150 et al., 2019; Takemura et al., 2017; Yandrapalli et al., 2016), we then tested if Gag and IRSp53
151 could associate directly or indirectly using immuno-precipitation assays (Fig 2a,b). Our results
152 showed that immuno-precipitation (IP) of endogenous IRSp53 resulted in co-precipitation of Gag
153 (Fig 2a, lane 1) as compared to controls (lanes 2 to 4). Unfortunately, we could not assess the
154 amount of IRSp53 pulled down by the antibody between conditions because the IgG signal
155 masked the endogenous IRSp53 signal. To overcome this issue, we performed an IP/co-IP
156 experiment of ectopic IRSp53-GFP/HIV-1 Gag proteins with an anti-GFP antibody, and
157 confirmed the pull down of IRSp53-GFP (Fig 2b, lanes 2 and 3) and the co-precipitation of Gag
158 (lane 3) while nothing was detected in the controls (lanes 1 and 4). Input and flowthrough were in
159 accordance with the results, showing less IRSp53-GFP in the flowthrough (lane 3). We then
160 concluded that HIV-1 Gag and endogenous IRSp53, or ectopic IRSp53-GFP, were components of
161 the same intracellular complex, interacting directly or indirectly through other factors or
162 membrane domains. IRSp53 is a cellular protein that switches from the cytosol to the cell plasma
163 membrane for inducing membrane ruffles upon activation by Rac1 and its effectors (Miki and
164 Takenawa, 2002; Suetsugu et al., 2006). We have previously shown that Gag cellular expression
165 triggers Rac1 activation (Thomas et al., 2015), on which IRSp53 membrane localization and
166 function is dependent. Thus, here, we compared the relative membrane binding of IRSp53 upon
167 cellular expression of HIV-1 Gag using membrane flotation assays (Fig 2c,d). In the absence of
168 Gag (“HEK293T control cells”), one could observe the presence of IRSp53 both in the cytosol
169 (Fig 2c, fractions 6-8, labeled with the ribosomal S6 biomarker) and in the cell membrane
170 fractions (Fig 2c, fractions 1-3, labeled with the membrane Lamp2 biomarker). The lysosomal
171 membrane protein Lamp2 and the cytosolic ribosomal protein S6 were used as controls to validate
172 the correct separation of the membrane and cytosolic fractions. Thus, at equilibrium, 19±8% of

IRSp53 was bound to cell membranes (Graph, Fig 2d). The same experiment was repeated with cells expressing HIV-1 Gag, where $66\pm 9\%$ of Gag was bound to the cell membranes (Graph, Fig 2d). Notably, we observed a 2-fold increase with $44\pm 5\%$ of IRSp53 bound to the cell membrane upon HIV-1 Gag expression (Graph Fig 2d) ($n=5$ independent experiments, p value = 0.000129, Student's t -test), while none with IRTKS. This effect was comparable with the one of Tsg101, a protein of the ESCRT-I complex known to interact mainly with the p6 domain of Gag (Garrus et al., 2001; Pornillos et al., 2003; von Schwedler et al., 2003) and partially with the NC domain (El Meshri et al., 2018). The cellular endosomal sorting complex required for transport (ESCRT) machinery has been involved in the mechanism of vesicular budding of intracellular multi-vesicular bodies, and also hijacked by the HIV-1 Gag protein for viral particle budding. Here, we observe a 2-fold increase in cell membrane binding of Tsg101 upon Gag expression, passing from $36\pm 10\%$ without Gag to $79\pm 8\%$ in the presence of Gag (Fig 2c) ($n=5$ independent experiments, p value = 0.00517, Student's t -test).

Furthermore, we examined if Gag/IRSp53 complexation was dependent on the p6 domain of Gag to reveal if this could be independent of ESCRT recruitment by Gag. We thus used a C-terminal mutant of Gag, Gag Δ p6, which is deficient in ESCRT-Tsg101 recruitment (von Schwedler et al., 2003), but is still capable of binding the plasma membrane and assembling particles that poorly bud. Gag Δ p6 viral particles are tethered and remain attached to the plasma membrane (see Floderer et al., 2018 for the characterization of Gag(i)mEos2 Δ p6). Our experiments revealed that Gag, Gag(i)mEos2 and Gag Δ p6(i)mEos2 were all pulled down with IRSp53 (Fig 2-Fig S1), showing that the addition of the internal mEos2 protein - a necessary tag for super resolution microscopy (SRM) imaging of Gag (see the following section) - did not affect the complexation of Gag with IRSp53. This was to show that the Gag used in the SRM imaging studies behaved similarly to wild-type Gag. Moreover, we showed that the p6 domain was not required for Gag/IRSp53 molecular interplay suggesting that it occurs before ESCRT recruitment.

198 Together, these results suggest that there is a complexation between HIV-1 Gag and IRSp53
199 reinforcing the idea of a strong molecular interplay between these two proteins directly or
200 indirectly but in the same membrane domain. We observed that cellular Gag expression, possibly
201 by triggering Rac1 activation (Thomas et al., 2015), favors cell membrane binding of IRSp53.

202

203 **3. Single Molecule Localization Microscopy reveals IRSp53 surrounding HIV-1 Gag**
204 **assembly sites.**

205 Our finding that IRSp53 and HIV-1 Gag are present in the same molecular complex at the cell
206 membrane motivated us to assess whether IRSp53 was present specifically at the Gag assembly
207 sites. Because HIV-1 assembly are ~100 nm in diameter (Floderer et al., 2018; Manley et al.,
208 2008), we used PALM (Photo-Activated Localization Microscopy) coupled to dSTORM (direct
209 Stochastic Optical Reconstruction Microscopy) with TIRF illumination, to investigate with high
210 precision the localization of I-BAR proteins in Gag(i)mEos2 assembly sites at the plasma
211 membrane (Fig 3, Fig 3- Fig S2, Fig 3- Fig S3).

212 We first checked our ability to correctly identify on going assembly sites by quantifying the
213 sizes of Gag clusters. For this we used purified HIV-1 Gag(i)mEos2 virus like particles (Fig 3-
214 FigS1b). When using the same size estimation method described in (Floderer et al., 2018), we
215 measured individual VLP sizes around 130-140 nm (Fig 3-FigS1b). VLP size was correctly
216 estimated (114+/-37 nm, median+/- 1st IQR) from the size distribution of 800 different clusters
217 identified in our VLP images using a density-based spatial scan (DBSCAN) clustering method
218 (Fig 3-FigS1b). Finally, DBSCAN showed us that these HIV-1 Gag(i)mEos2 cluster size
219 distributions had similar median values for each cell type in each condition (HEK (96 +/- 44nm,
220 Fig 3-FigS1c) or Jurkat T cells (105 +/- 66nm, FigS5d) when IRSp53 was immuno-labelled, HEK
221 (116 +/- 68nm, Fig 3-FigS1e) or Jurkat T cells (96 +/- 50nm, FigS5f) when IRTKS was immuno-

222 labelled), allowing us to directly compare IRSp53 and IRTKS organization close to these
223 assembly sites.

224 In HEK293T cells and in Jurkat T cells, reconstructed dual color PALM/STORM images
225 exhibited Gag(i)mEos2 assembly sites close to or overlapping with IRSp53 (Fig 3a, e, S6, S7)
226 whereas Gag clusters did not seem to overlap with IRTKS (Fig 3b, f, S6, S7). These results are
227 consistent with the siRNA data presented in Fig. 1b. In order to quantify these observations, we
228 performed coordinate-based colocalization (Malkusch et al., 2012) (CBC) analysis of HIV-1 Gag
229 and IRSp53 (or IRTKS) in close proximity of HIV-1 assembly sites. We first isolated the
230 assembly sites by segmentation and retrieved their centre positions. We then kept all HIV-1 Gag
231 located within a distance of 80 nm from this centre (70 to 80% of all cluster sizes measured by
232 DBSCAN are found within this distance) and all the IRSp53 (or IRTKS) are found in a distance
233 of 150 nm from this centre (~2x the assembly site size, see Fig 3-Fig S4 for details on the process
234 workflow). We choose this IRSp53 (or IRTKS) cut-off distance to minimize the contribution of
235 cross-colocalization between different HIV-1 Gag clusters. In contrast to classical colocalization
236 analysis, CBC takes into account the spatial distribution of biomolecules to avoid excessive
237 colocalization due to local densities of single molecules and provides a colocalization value for
238 each single-molecule localization. This CBC value ranges from -1 to +1, where -1 corresponds to
239 anti-correlation, 0 indicating non-correlation and +1 corresponds to perfect correlation between
240 the two molecules. Since CBC values are calculated for each localization, we plotted the CBC
241 values as frequency distributions for all localizations of IRSp53/Gag and IRTKS/Gag. As shown
242 in Fig. 3c (for n=4 HEK293T cells) or in Fig 3g (for n=5 Jurkat T-cells), the CBC distribution for
243 IRSp53/Gag has a higher proportion of values exhibiting high colocalization (27% (in HEK293T
244 cells) or 26% (in Jurkat T-cells) of $CBC > 0.5$) in comparison to IRTKS/Gag values (14% (both
245 cell types, n=5 for HEK293T and Jurkat T-cells) of $CBC > 0.5$) which instead show a very high
246 proportion (close to 75%) of anti-correlation ($CBC < 0$) (Fig 3d,h). This comparison directly shows

247 that IRSp53 displays stronger single molecule colocalization with Gag in assembling clusters than
248 IRTKS does.

249 Although CBC values give a quantitative value of the colocalization, it does not provide direct
250 information on the average positions of IRTKS or IRSp53 molecules with respect to Gag
251 molecules within the assembling clusters. Moreover, as for all the colocalisation methods use to
252 analyze molecule proximities, the CBC method is a strongly parameterize method which results
253 can depend on the parameter values (total distance of search for colocalisation, number of
254 searching circles...). Thus, to gain more insight into these colocalisation quantification, we
255 performed simulations to generate different patterns of PALM/STORM localizations (Fig 4), and
256 analyzed them with the same set of parameters (total distance and distance steps) that the one we
257 used for the experimental data. Since IRSp53 and IRTKS are membrane curvature sensors and
258 since we observed that a lack of IRSp53 stops the assembling bud at half completion (Fig 1e), we
259 hypothesis that they will be located around the on-going assembly site. We therefore simulated an
260 annulus of IRSp53 or IRTKS STORM localizations with different waists located at increasing
261 distances from the centre of Gag clusters (Fig 4a and Fig 4-Fig S1). Numerically generated
262 images were then analyzed identically to our experimental images (see methods for details) and
263 CBC distributions were generated (Fig 4b). Experimental and simulated cumulative distribution
264 functions of the CBC values were compared by performing a root mean square error (RMSE)
265 quantification (Fig 4-Fig S1, g,h,i,j). The lowest RMSE value was considered as the best
266 similarity between the two distributions. This comparison indicated that IRSp53 localization, on
267 average, displays a restricted pattern around and in the assembly sites. This corresponds to a
268 circular ring surrounding the assembly site at 80 nm from the centre of the Gag budding sites with
269 a width of 80 nm (Fig 4 c,e). On the other hand, IRTKS was present as a large diffuse pattern
270 centered at 140 nm from the Gag assembly site centre with a width of 200 nm, explaining why
271 fewer IRTKS molecules were detected in the assembly sites (Fig 4 d,f). Interestingly, the maps of

the RMSE values show that, on average the surrounding belt centre (independently of its waist) is located nearer to the HIV-1 Gag assembly centre in the case of IRSp53 as compared to that of IRTKS (Fig 4-Fig S1 g,h,i,j). Our results thus show that IRSp53 indeed specifically localizes at HIV-1 Gag assembly sites at the cell plasma membrane, whereas IRTKS poorly does.

The involvement of IRSp53 around Gag assembly sites seems to be conserved regardless of the cell type, reinforcing the idea of a specific role for IRSp53 in HIV-1 Gag particle assembly.

4. IRSp53 is incorporated in HIV-1 particles.

To assess IRSp53 incorporation into HIV-1 Gag particles, we purified Gag virus-like particles (Gag-VLP) from cells transfected with Gag-mCherry and several GFP-tagged I-BAR domain proteins (Fig 5a). The IRSp53-I-BAR-GFP construct only contains the membrane curving I-BAR domain of IRSp53. PH-PLC δ -GFP, a PI(4,5)P₂ binding protein, was used as a control, because it binds PI(4,5)P₂ but does not generate membrane curvature. Fluorescent VLPs were purified from these transfected cells, then visualized for two colors (green: GFP and red: mCherry), and then a Mander's coefficient was calculated as an indicator of incorporation of the ectopic (green) GFP-tagged proteins within the (red) Gag-mCherry VLPs and vice-versa (see Materials and Methods) (Fig 5 a,b). We found a high correlation (Mander's coefficient = 0.95-1) between IRSp53-GFP and Gag-mCherry (Fig 3b, Left Graph, Red Column), indicating that almost all Gag-mCherry VLPs contained IRSp53-GFP, while the reverse (Green Column) was not true, indicating that all the IRSp53-GFP-labelled vesicles produced by the cells were not all positive for Gag . When using the IRSp53-I-BAR domain alone, we also obtained a high Mander's coefficient, ~0.8 (Fig 5b). In contrast, for IRTKS-GFP, the Mander's coefficient was 0.4-0.5, indicating no significant correlation between IRTKS-GFP and Gag-mCherry. This indicates that even if a domain (PH-PLC δ) or another I-BAR protein (IRTKS) can recognize PI(4,5)P₂ at the cell membrane, it is not sufficient for incorporation into Gag-VLPs. Finally, we found that the reverse correlation of GFP-

297 tagged proteins with Gag-mCherry was under the random 0.5 coefficient (Fig 5b, right Graph,
298 Green Column), indicating that, on average, GFP-tagged vesicles produced by the cells did not
299 contain Gag. Taken together, these results show the preferential incorporation of IRSp53-GFP
300 into released HIV-1 Gag-mCherry virus-like- particles.

301 To study the incorporation of endogenous IRSp53 in HIV-1 particles, cells were transfected
302 with plasmids expressing either wild-type infectious HIV-1 or codon optimized immature HIV-1
303 Gag protein (without genomic RNA). The virus particles were purified through a 20%-sucrose
304 cushion or further through a continuous iodixanol density gradient (as in (Grigorov et al., 2009,
305 2006)). IRSp53 was found to be associated with the viral particles in both conditions, i.e. in
306 infectious HIV-1 and in Gag VLPs, indicating that Gag alone is sufficient to recruit IRSp53 in the
307 viral particles (Fig 5c,d, “IRSp53”). Tsg101 also showed an association with viral particles in
308 both conditions (Fig 5c,d, “Tsg101”), as reported previously (Garrus et al., 2001; Hammarstedt
309 and Garoff, 2004; Pornillos et al., 2003). In contrast, IRTKS was not associated neither with Gag-
310 VLP nor HIV-1 particles (Fig 5c,d, “IRTKS”). Upon further purification (Fig. 5e), IRSp53, and
311 the ESCRT proteins, Tsg101 and ALIX, were found associated within the same fractions
312 containing the HIV-1 Gag viral particles, together with other well-known viral particle cofactors
313 such as CD81, CD63 tetraspanins (Grigorov et al., 2009, 2006). Thus, endogenous IRSp53 is
314 most probably incorporated in HIV-1 particles in a Gag dependent manner.

315

316 5. HIV-1 Gag is enriched at membrane tube tips generated by IRSp53 I-BAR domain.

317 The results above demonstrate that not only is IRSp53 incorporated in Gag-VLPs, but it is
318 present at the budding sites and its deletion strongly reduces HIV-1 particle release in a Gag-
319 dependent manner by arresting its bud assembly at the cell plasma membrane (Fig 1). In order to
320 advance molecular mechanistic understanding of the role of IRSp53 locally at Gag assembly sites,
321 we assessed IRSp53 I-BAR/Gag interplay on model membranes giant unilamellar vesicles

(GUVs). Previous in vitro studies showed that when placing IRSp53 I-BAR domain outside PI(4,5)P₂-containing GUVs, the I-BAR domain can deform GUV membranes, generating tubes towards the vesicle interior (Jarin et al., 2019; Prévost et al., 2015; Saarikangas et al., 2009). Consistent with the previous observations, we observed I-BAR driven tubulations on GUVs at low I-BAR concentrations (0.005 – 0.06 μ M, see Fig. 6- Fig S1c as an example); surprisingly, when increasing I-BAR concentrations to 0.1 μ M and up to 1 μ M, we observed a decrease in the number of GUVs having tubes. Future work is required to investigate this seemingly puzzling observation.

For our Gag membrane binding assay, we first used a high concentration of IRSp53 I-BAR domain (0.5 μ M), while keeping the PI(4,5)P₂ concentration constant. This was done in order to prevent the generation of tubes by IRSp53 I-BAR domain and to focus on analyzing the membrane binding efficiency of Gag in the presence of IRSp53 I-BAR domain on *flat*, non-deformed GUVs. We found that Gag binding to GUV membranes is increased ~7 fold when IRSp53-I-BAR domain was introduced first on GUVs before adding Gag (median value 6.7), compared to the condition of Gag only (median value 0.9) ($p < 0.0001$, Student's *t*-test) (Fig 6a, "Gag only" vs. "I-BAR+Gag"). However, in the condition where Gag was introduced before adding the I-BAR domain, Gag intensity on GUV membranes increased only ~3 fold as compared to the Gag only condition ($p < 0.0001$, Student's *t*-test) (Fig 6a, "Gag only" vs. "Gag+I-BAR"). Notably, by comparing I-BAR+Gag and Gag+I-BAR conditions, we observed a 2-fold higher Gag intensity on GUV membranes in the first condition ($p < 0.0001$, Student's *t*-test) (Fig 6a, "I-BAR+Gag" vs. "Gag+I-BAR"). Taken together, these results show that IRSp53 I-BAR domain facilitates Gag membrane binding on GUV in favor of increasing Gag concentration locally. Furthermore, given that in our cell experiments, we observed a relative increase of IRSp53 bound to the cell membranes upon HIV-1 Gag expression (Fig. 2d), we also assessed if HIV-1 Gag could facilitate the membrane binding of IRSp53 I-BAR domain. Consistent with our cell

347 experiment results, we observed a similar increase of the membrane binding (~ 1.5 fold) of
348 IRSp53 I-BAR domain in the presence of HIV-1 Gag on GUVs (Fig 6-Fig. S1 a,b) suggesting a
349 strong interplay between Gag and IRSp53 I-BAR domain.

350 Given these results, and that IRSp53 is a membrane curving protein involved in the early
351 stages of cell protrusion generation (Disanza et al., 2013; Sathe et al., 2018), we asked whether
352 the local membrane deformation induced by IRSp53 could be a preferred location for HIV-1 Gag
353 assembly. We incubated GUVs with IRSp53 I-BAR domain at a low concentration (0.05 μ M),
354 which allows for the generation of inward membrane tubes, followed by the addition of Gag (Fig
355 6 b, see Materials and Methods). This experiment revealed that Gag was sorted preferentially to
356 the tips of the tubes generated by the IRSp53 I-BAR domain (Fig 6 b,c,d, Video 1 and Fig 6-Fig
357 S1 c,d). We note that the majority of the tubes in GUVs were moving too rapidly, preventing us
358 from acquiring images with high spatial resolution (see Videos 2 and 3). However, we observed
359 that the Gag signals appeared dotted inside GUVs (Fig 6-Fig. S2 and Videos 2 and 3), which is
360 very different from the IRSp53 I-BAR domain signal that is clearly all along the tubes (Fig 6-Fig.
361 S1 c). Moreover, for tubes that were not moving too fast, we found that for all the tubes (17 tubes,
362 protein concentrations: 0.05 μ M unlabeled I-BAR domain and 0.3 μ M AX488 Gag), the Gag
363 signal was exclusively located at the tips of the tubes (Fig. 6d and Video 1). Finally, we observed
364 that addition of HIV-1 Gag resulted in the formation of shorter I-BAR tubules as compared to
365 GUVs incubated with the IRSp53-I-BAR domain alone (Fig 6-Fig S1 c,d), indicating an
366 interference in I-BAR tubule elongation when Gag sorted to the tubule tips, suggesting that Gag
367 usurps the IRSp53 tubulation function.

368 Together, these results demonstrate that HIV-1 Gag binding to membranes is enhanced
369 locally by the presence of IRSp53 I-BAR domain, and that Gag preferentially binds to highly
370 curved membranes generated by the I-BAR domain of IRSp53.

371

372 **Discussion**

373 The findings of this study uncovered the role of the host cellular I-BAR factor IRSp53 in
374 HIV-1 Gag assembly and membrane curvature upon bud formation. In vitro, on GUVs, we
375 showed that the IRSp53 I-BAR domain enhances Gag membrane binding locally (Fig 6), and vice
376 versa (Fig 6-Fig S1 a,b), in agreement with cell membrane flotation assays also showing an
377 increase of IRSp53 membrane retention upon Gag expression (Fig 2). Indeed, IRSp53 was found
378 at, or in close vicinity to Gag assembly platforms at the cell membrane (Figs 3, 4), and is
379 incorporated into Gag virus-like particles and in HIV-1 virions (Fig 5). Importantly, we revealed
380 that the partial knockdown of IRSp53 gene expression arrested Gag assembly at the mid-bud
381 formation stage (Fig 1) and that Gag preferentially localizes at the tube tips induced by IRSp53 I-
382 BAR domain, interfering with its long tubule formation in vitro (Fig 6). Altogether, IRSp53
383 appears instrumental in membrane curvature upon HIV-1 budding and is locally subverted as an
384 essential factor needed for full HIV-1 Gag particle assembly.

385 Using GUVs, we observed that Gag not only colocalizes with IRSp53 I-BAR domain on
386 vesicles, but that the IRSp53-I-BAR domain increases Gag binding to these model membranes,
387 mimicking the possible local Gag/IRSp53 interplay at the assembly site (Fig 6a). Indeed, BAR
388 domain proteins, in general, and IRSp53 in particular, are known to induce strong PI(4,5)P₂
389 clusters (Saarikangas et al., 2009; Zhao et al., 2013); PI(4,5)P₂ was shown to play a role in Gag
390 binding to the cell plasma membrane (Ono et al., 2004), as well as PI(4,5)P₂ is strongly clustered
391 during virus assembly (Favard et al., 2019; Ono et al., 2004; Yandrapalli et al., 2016). Thus, these
392 results suggest that the membrane binding of Gag on IRSp53-enriched membrane domains could
393 promote the plasma membrane binding of both proteins (Fig 3). This is in agreement with super
394 resolution imaging in cells, where Gag/IRSp53 interactions may take place at the Gag assembly
395 sites as IRSp53 was localized in close proximity to Gag assembly sites in both HEK293T cells
396 and CD4 Jurkat T cells (Fig 3). Our experiments suggest that Gag and IRSp53 are associated in a

397 common complex at the cell plasma membrane (Fig 2). Given that upon Gag expression IRSp53
398 increases its binding to the cell membrane (Fig 2), this suggests that Gag could activate IRSp53
399 through Rac1 activation (Thomas et al., 2015) or perhaps by releasing its auto-inhibition (Kast
400 and Dominguez, 2019). However, these explanations remain to be tested.

401 HIV-1 particles are known to incorporate a large number of cellular proteins, many of which
402 are directly involved in virus budding (Hammarstedt and Garoff, 2004). Here, we showed that
403 IRSp53 is incorporated in Gag-VLPs, as well as in purified HIV-1 virions (Fig 5), which most
404 likely depends on the I-BAR domain of IRSp53 (Fig 5). Using the IRSp53-I-BAR domain on
405 GUVs, we induced membrane protrusions that have a negative mean curvature similar to a viral
406 bud; Gag was found particularly at the tube tips that have a half-sphere geometry similar to a viral
407 bud (Fig 6, Videos 1, 2, 3). This indicates that Gag binds preferentially to IRSp53 I-BAR-curved
408 membranes *in vitro* in contrast to other almost-flat areas of the GUVs. Similarly, in cells, single
409 molecule localisation images reveal some Gag clusters enriched at IRSp53 labelled protrusions at
410 the plasma membrane (Fig 3-Fig S2). IRSp53 clusters have already been reported prior to
411 filopodia formation (Disanza et al., 2013) and in negatively curved area at the onset of endocytic
412 buds (Sathe et al., 2018). Moreover, it was shown that inducing local membrane curvature helps
413 to initiate Gag lattice formation (Pak et al., 2017). We thus propose that IRSp53 induces local
414 membrane curvature, most likely upon activation through Rac1/Cdc42 and effectors, which in
415 turn can promote local Gag recruitment and initiation of the viral assembly (knowing that
416 expression of Gag can activate Rac1 (Thomas et al., 2015)). This appears to be independent of the
417 cell types (Fig 3).

418 Although the presence of RNA can facilitate the growth of the Gag network (Chen et al.,
419 2014; Floderer et al., 2018), favouring membrane bending due to the intrinsic curvature of
420 assembling Gag hexamers, coarse grained simulations of HIV-1 Gag assembly showed that,
421 above a certain threshold, this Gag self-assembly is unable to overcome the free energy penalty

422 required to curve the membrane. Here, we observed that siRNA knockdown of IRSp53 gene
423 expression induces a decrease in viral particle production and arrests the assembly at half
424 completion (Fig 1). Since IRSp53 stabilizes curvature by scaffolding (Prévost et al., 2015),
425 another role of IRSp53 could be to lower this free energy barrier involved in the progression of
426 the budding process beyond the half-sphere geometry by stabilizing long enough the bud
427 curvature. This stabilization could be achieved either directly by organizing linearly around the
428 assembly site (Jarin et al., 2019) and mechanically constricting the nascent bud, or indirectly with
429 the help of actin polymerization. Interestingly, Ku *et al.*, also observed that 60% of assembling
430 particles exhibit a pause around the midway mark of the assembly process (Ku et al., 2013). This
431 pause can provide a temporal window for IRSp53 to intervene in the progression of HIV-1
432 particle assembly as we propose here.

433 Finally, ESCRT recruitment occurs at the end of virus assembly, after the membrane has been
434 curved, forming a vesicle ready to bud (Bleck et al., 2014; Johnson et al., 2018). Overexpression
435 of a mutant of the ESCRT protein Tsg101 was previously shown to block HIV-1 budding at a late
436 stage, arresting the budding with a characteristic bulb shaped phenotype indicative of a defect in
437 the late stage of the bud scission (Goila-Gaur et al., 2003), in contrast with our observations with
438 the IRSp53 siRNA phenotype (Fig 1, Fig 1-Fig S3). Consequently, this suggests that Gag-IRSp53
439 association is ESCRT independent (as shown in Fig 2-Fig S1) and occurs at an earlier stage of
440 virus assembly.

441 Another study (Mercenne et al., 2015) showed that angiomin, which acts as an adaptor
442 protein for HIV-1 Gag and the ubiquitin ligase NEDD4L, functions in HIV-1 assembly prior to
443 ESCRT-I recruitment. Interestingly, angiomin also contains a BAR domain (Moleirinho et al.,
444 2014), but it is canonically involved in inducing *positive* curvature, as opposed to the *negative*
445 curvature induced by I-BAR IRSp53. Thus, it is possible that angiomin functions in another

way, for example, at the viral bud neck which has both positive and negative curvatures by facilitating ESCRT recruitment.

IRSp53 itself is a scaffold protein for cofactors of cortical actin signalling (Zhao et al., 2011) and we have previously shown that a Rac1 signalling pathway, including IRSp53, is involved in HIV-1 particle production (Thomas et al., 2015). Thus, it is possible that IRSp53 could also play a role in generating local cortical actin density in the vicinity of the viral bud in formation. The role of cortical actin associated with IRSp53 scaffolding in that context remains to be elucidated.

Our work illustrates a novel role for the host cellular I-BAR factor IRSp53, which is subverted by the retroviral Gag protein, in HIV-1-induced membrane curvature and in favoring the formation of the fully assembled viral particle.

Materials and Methods

Antibodies.

A rabbit polyclonal anti-IRSp53 antibody (Merck Millipore), a rabbit polyclonal anti-IRTKS (Bethyl), a mouse monoclonal anti-CA (NIH AIDS Reagent Program), a rabbit polyclonal anti-GFP (Invitrogen), a mouse monoclonal anti human CD63 (Santa Cruz biotechnology), a mouse monoclonal anti human CD81 (Santa Cruz biotechnology), a rabbit monoclonal anti-human TSG101 (Abcam), and a rabbit polyclonal anti human ALIX (Covalab) and secondary anti-rabbit Atto647N antibody (Sigma) were used in this study.

Plasmids.

469 The plasmid expressing HIV-1 codon optimized Gag (pCMVGag(myc) [named pGag]), the
470 plasmid expressing Pol and Env-deleted HIV-1 (named pNL4.3ΔPolΔEnv was a gift of E.Freed,
471 HIVDRP, NIH, USA) encoding Gag alone with its packageable viral RNA (A. K. Chen et al.,
472 2014) and the plasmid expressing full wild-type HIV-1 (named pNL4.3) were described
473 previously (Favard et al., 2019). Plasmids IRSp53-GFP, IRTKS-GFP, PinkBAR-GFP and
474 IRSp53-I-BAR-GFP were obtained from University of Helsinki (Finland) (Saarikangas et al.,
475 2009). Plasmids expressing PH-PLCδ-GFP was a gift of B.Beaumelle (IRIM, France),
476 Gag(i)mCherry (named Gag-mCherry), Gag tagged with internal photo-activable mEos2 (named
477 Gag(i)mEos2), p6-deleted Gag tagged with mEos2 (named pGagΔp6-mEos2) were described in
478 (Floderer et al., 2018).

479 **siRNA.**

480 Stealth siRNA (Invitrogen) targeting IRSp53 (BAIAP2) and IRTKS (BAIAP2L2), and
481 Smartpools (Dharmacon) targeting IRSp53 (BAIAP2) or random sequence for siRNA controls
482 were used in this study.

483 **Cell culture and transfection.**

484 Human embryonic kidney cells (HEK 293T-ATCC-CRL-1575TM) were maintained in
485 Dulbecco's Modified Eagle's Medium (DMEM, GIBCO) and Human Jurkat T-lymphocytes
486 (ATCC-CRL-2899TM) were maintained in RPMI (GIBCO). All cell lines were tested negative
487 for mycoplasma thanks to a MycoAlertTM Mycoplasma Detection Kit (Lonza Bioscience) done
488 every month. Media was supplemented with 10% fetal bovine serum (FBS, Dominique Dutscher)
489 and complemented with sodium pyruvate and antibiotics (penicillin-streptomycin). Cells were
490 grown at 37°C in a5% CO₂ atmosphere. Transfection was performed by using the calcium
491 phosphate precipitate method on HEK293T cells (as described in (Grigorov et al., 2006) and the
492 AMAXA (Lonza) method according to the manufacturer's instructions for the Jurkat T cells (as in
493 (Thomas et al., 2015)). Based on different plasmids conditions, cells were transfected (2x10⁶

cells/transfection) with a total of 8µg of plasmids. The amount of transfected plasmid was normalized by adding pcDNA3.1 empty plasmid DNA. Cell medium was replaced by fresh medium 6 hours post-transfection and experiments were performed 24-48h post transfection. SiRNA transfections in cells were performed with either RNAiMax (Invitrogen) or calcium phosphate buffer in HEK293T cells or by electroporation for Jurkat T cells. One day prior to transfection, 2×10^5 cells/well were seeded in 2mL of growth medium without antibiotics, in a 6-well plate. Transfection was performed using the manufacturer's protocol. 24 hours after siRNA transfection, the cells were again transfected using the phosphate calcium buffer method. These cells were incubated at 37°C in a 5% CO₂ atmosphere, for 24/48 hours.

Immunoprecipitation assay.

Based on different plasmid conditions, HEK293T cells (2×10^6 cells) were transfected with pGag alone or pGag/pIRSp53-GFP plasmids (8µg total) and the amount of transfected plasmid was normalized by adding pcDNA3.1 “mock” plasmid. The cell medium was replaced 6 hours post-transfection. Twenty-four hours post-transfection, the cells were washed with cold 1X-Phosphate Buffer Solution (PBS) prior to collection with 800µL of chilled lysis buffer (50mM TRIS-HCl [pH=7.4]; 150mM NaCl; 1mM EDTA; 1mM CaCl₂; 1mM MgCl₂; 1% Triton, 0.5% sodium deoxycholate; protease inhibitor cocktail [Roche] 1 tablet/10mL lysis buffer). The cells were incubated on ice for 30 minutes and then centrifuged at 13,000 rpm/ 15minutes/ 4°C. The supernatant was collected in a new tube and the pellet was discarded. For each condition, 1000µg of protein (the collected supernatant) was incubated with 1µg of anti-IRSp53 or anti-GFP antibody on a tube rotator overnight at 4°C. 25µL of beads (Dynabeads Protein A, Life Technologies) was added to each tube of protein-antibody complex and incubated for 2 hours on the tube rotator at 4°C. The samples were then washed 5 times with the lysis buffer, followed by addition of 20µL 2X Laemmli's buffer to the beads. The samples were denatured at 95°C for 10 minutes and then processed for Western blot.

519 **Western blot and analysis.**

520 50µg of each protein (intracellular in cell lysates) samples or 20µL of purified VLP samples, were
521 mixed with SDS loading dye, deposited and resolved on a 10% SDS-PAGE gel. The gels were
522 then transferred on to polyvinylidene PVDF membranes (Amersham). Immunoblotting was
523 performed by incubating the membranes overnight with primary antibody at 4°C, and 2 hours
524 with Horseradish-Peroxydase (HRP)-conjugated secondary antibody at room temperature. The
525 western blot signals were detected using ECL Prime/ECL Select substrate (Amersham) and
526 images were taken using ChemiDoc (BioRad).

527 **VLP purification and quantification.**

528 Twenty-four or 48 hours post-transfection, cell culture supernatants containing Gag-VLPs were
529 collected, filtered through a 0.45µm filter, and clarified at 800xg for 5min at 4°C. The supernatant
530 was then purified by loading it on a cushion of 25% sucrose in TNE buffer (25mM Tris-HCl,
531 4mM EDTA, 150mM NaCl) and ultracentrifuged at 100000xg for 1h30 at 4°C in a SW41Ti rotor
532 (Beckman Coulter). Dry pellets were resuspended in TNE buffer at 4°C overnight. Gag-VLP
533 release was estimated by performing anti-CAP24 immunoblot and by quantifying Gag signal in
534 the blots using Fiji software as described in (Thomas et al., 2015). The calculation for Gag-VLP
535 release is: % of Gag in VLP = $\text{Gag}_{\text{released}} / (\text{Gag}_{\text{released}} + \text{Gag}_{\text{intracellular normalized to GAPDH}})$.

536 **Membrane flotation assay.**

537 For each condition, 4×10^6 cells were transfected and viral supernatants were harvested 48h post-
538 transfection, as described above. Cells were washed with ice-cold PBS and resuspended in Tris-
539 HCl containing 4mM EDTA and 1x complete protease inhibitor cocktail (Roche). Every step was
540 then performed at 4°C. Cell suspensions were lysed using a dounce homogenizer, then
541 centrifuged at 600g for 3min to obtain Post-Nuclear Supernatants (PNS). A cushion of 820µL of
542 75% (wt/vol) sucrose in TNE buffer was loaded at the bottom of an ultracentrifuge tube and
543 mixed with 180 µL of PNS adjusted to 150mM NaCl. Two milliliters and 300 µL of 50% (wt/ml)

544 sucrose cushion followed by 0.9 mL of 10% (wt/ml) sucrose cushion were then layered to obtain
545 the gradient that was then centrifuged in a Beckmann SW60Ti rotor at 35 000rpm, 4°C,
546 overnight. Eight fractions of 500μL were collected from the top to the bottom and analyzed by
547 western blotting.

548 **Transmission electron microscopy.**

549 SiRNA treated HEK293T cells were fixed in 4% paraformaldehyde and 1% glutaraldehyde in
550 0.1M phosphate buffer (pH 7.2) for 48h, washed with PBS, post-fixed in 1% osmium tetroxide for
551 1h and dehydrated in a graded series of ethanol solutions. Cell pellets were embedded in EPON™
552 resin (Sigma) that was allowed to polymerize at 60°C for 48h. Ultrathin sections were cut, stained
553 with 5% uranyl acetate and 5% lead citrate and deposited on colloidon-coated EM grids for
554 examination using a JEOL 1230 transmission electron microscope.

555 **Sample preparation for super resolution PALM/STORM microscopy.**

556 HEK293T cells expressing HIV-1 Gag/Gag(i)mEos2 cultured on poly-l-lysine (Sigma) coated 25
557 mm round #1.5 coverslips (VWR) were fixed using 4%PFA + 4% sucrose in PBS for 15 min at
558 room temperature. Samples were subsequently quenched in 50mM NH₄Cl for 5 min. Samples
559 were then washed in PBS and then blocked for 15 min in room temperature using 1%BSA in PBS
560 and subsequently in 0.05% Saponin in 1%BSA in PBS. Samples were stained using a 1:100
561 dilution of the primary antibodies (rabbit polyclonal anti-human IRSp53, Sigma and rabbit
562 polyclonal anti-human IRTKS antibody, Bethyl) for 60 min in room temperature. Samples were
563 washed 3x 5min using 1% BSA in PBS followed by 60 min staining using a 1:2000 dilution of
564 the anti-rabbit Atto647N antibody (Sigma). Samples were washed 3x5 min with PBS and stored
565 in light protected container in +4°C until imaged. Samples were mounted on a StarFrost slide with
566 a silicon joint with the STORM buffer (Abbelight). Cells were imaged within 60 minutes after
567 application of the STORM buffer.

568 **PALM/STORM Imaging.**

Single-molecule localization microscopy was performed on a Nikon inverted microscope equipped with 405-, 488-, 561- and 642-nm lasers, an EMCCD Evolve 512 Photometrics camera (512*512, 16µm pixel size) with an oil immersion objective 100X NA1.49 Plan Apochromat. PALM imaging of Gag mEos2, activation was performed with lasers irradiance set to 0.3 kW/cm² for 405 nm conversation and ~ 2.2 kW/cm² for 561 nm excitation. Illumination was performed over a 25x25 µm area in the sample (1/e² spatial irradiance distance) in TIRF-mode. 20-50,000 images were acquired for each cell with 50 ms integration time. The mean precision localisation in PALM measurements was found to be 20±5 nm (Fig 3-FigS1a). 2D-STORM imaging of Atto647N, was performed using a ~5 kW/cm² irradiance with the 642 nm excitation. 25000 images were acquired for each condition. Tetraspeck 100 nm multicolor beads (Life Technologies) as fiducial markers to correct for drift and chromatic abberation.

Single Molecule Localization Microscopy Image Reconstruction and Analysis.

SMLM acquisitions were analyzed using the ThunderSTORM plugin in FIJI (Ovesný et al., 2014). The mean precision localisation in PALM measurements was found to be 20±5 nm (mean±sd) and 27±9 nm for STORM (FigS5). During post-processing, a density filter was applied first to eliminate the background noise by identifying and discarding "isolated" localisations, with a threshold of a minimum of 5 neighbours in a 50 nm radius for a molecule to be considered "not isolated". In the next step, molecules that converged to the same position were identified as duplicates and removed, keeping the localization with the smallest uncertainty as the valid coordinate. Furthermore, molecules re-appearing with one "off-frame" within 20 nm were merged into one single localization, with the first one appearing considered as the valid localisation. Following these steps of post-processing, the sample drift was corrected by the drift correction module using fiducial markers described above. Each acquisition had at least 2 fiducial markers in the field of illumination. The resulting list of localisations was then used to reconstructing the respective PALM and STORM images and for the coordinate based

594 colocalisation (CBC) analyses using the Thunderstorm module. The module DBSCAN of the
595 super resolution quantification software SR Tessler (Levet et al., 2015) was used to analyse the
596 PALM localizations for quantification of Gag cluster sizes. In order to monitor the localisation of
597 I-BAR proteins in the vicinity of Gag assembling particles, the Gag particles were segmented by
598 thresholding using Fiji, to generate a binary mask of the PALM images. The centres of each Gag
599 assembling cluster were then determined and a custom MATLAB (Mathworks) code was used to
600 extract localizations in a radius of 80nm around each Gag cluster centre and to extract I-BAR
601 proteins localisations belonging to a disk of 150nm radius around the centre of each Gag clusters
602 (see Results section). These subsets of coordinates were then used to calculate the experimental
603 coordinate-based colocalization (CBC), with the algorithm developed by Malkusch et al.
604 (Malkusch et al., 2012) and implemented in the ThunderSTORM plugin of Fiji (see Fig. S8). The
605 coordinate-based colocalization (CBC) values are calculated from single-molecule localization
606 data of two species (Gag and IBAR proteins (IRSp53 or IRTKS)). A CBC value is assigned to
607 each single localization of each species. We analyzed the distributions of these CBC values by
608 plotting and comparing the distribution histograms of the CBCs obtained in the two conditions
609 (IRSp53 vs IRTKS).

610 We also performed a set of numerical simulations of super resolution images in Fiji. For this we
611 generated images by randomly choosing a molecule position within a radius of 60 nm from the
612 cluster centre for Gag (a compromise between the values found in the EM images (Fig.1e) and
613 those obtained by DBSCAN analysis in the SMLM experimental images (Fig. S5). For I-BAR
614 proteins we randomly assign positions within belts of different waists (from 0 to 100 nm) located
615 at different distances (from 0 to 160 nm) from the Gag cluster centre (see Fig.4 and Fig.S9). Each
616 localisation was then randomly assigned a brightness such that the final pointing precision
617 obtained at the end of the process for the Gag localisation was equivalent to the experimental one.
618 We then convolved each of these positions by a Gaussian 2D PSF, to generate a diffraction

619 limited image in which random noise was introduced using the “add noise” function of Fiji. Super
620 resolution images of these simulated localizations were then reconstructed using the
621 Thunderstorm plugin of Fiji with the same parameters as the one used in the experimental data
622 reconstruction. Finally, once the data set of localisations for both numerically simulated Gag
623 clusters and associated surrounding I-BAR proteins were obtained, they were analysed with the
624 CBC function of Thunderstorm with the same parameters as in the experimental analysis
625 (analysing all the neighbour positions within 10 successive radius of 20 nm each to estimate the
626 CBC). Each localization was attributed a CBC values and we compared the experimental
627 cumulative distribution functions of CBC values to the simulated cumulative distribution
628 functions using RMSE measurements, in order to establish the most probable configuration of I-
629 BAR belts around the on-going Gag assembly site.

630 **Preparation and Imaging of Fluorescent VLPs.**

631 Twenty-four hours after seeding, 2.10^6 HEK293T cells were transfected with 8µg of I-BAR-GFP
632 expressing plasmid with or without 8µg of pGag/pGag(i)mCherry plasmids (2/3 and 1/3
633 respectively). Twenty-four hours after transfection, cell media (9mL) was filtered before
634 performing VLPs purification by ultracentrifugation in a SW41Ti rotor (Beckman Coulter) at 29
635 000rpm, for 1h30, at 4°C, on a 20% sucrose cushion in TNE buffer. Dry pellets were resuspended
636 in 110µL of TNE and allowed to sediment on round 25mm coverslips during 45 minutes in a
637 AttoFluor Cell Chamber (Invitrogen). VLPs were imaged with a Nikon Ti Eclipse 2 TIRF
638 microscope. Images were taken with an Evolve EMCCD camera – 512 photometrics, using a
639 NA=1.45, 100X objective and using 488 and 561nm lasers.

640 **Image Analysis for Colocalization.**

641 Images were acquired with a Zeiss LSM780 (for fixed cells) or a Nikon Eclipse Ti-2 in TIRF
642 mode (for fluorescent viral particles). Colocalization analysis based on Mander’s coefficients was
643 performed using JaCOP (Just another Colocalization Plugin) (Bolte and Cordelières, 2006).

644 Mander's coefficients are defined as $M1 = \frac{\sum_i A_{i,coloc}}{\sum_i A_i}$ and $M2 = \frac{\sum_i B_{i,coloc}}{\sum_i B_i}$, A and B being the two
645 respective channels (mCherry and GFP). $0 < M < 1$, with 1 full colocalization and 0.5 random
646 colocalization. The M1 and M2 coefficients were calculated for several images and then
647 represented as column graphs with red columns representing the degree of overlap of mCherry
648 images with GFP images, and green columns representing the inverse.

649 **Iodixanol gradient.**

650 Cell culture medium of HEK293T (2.5×10^6 cells plated) transfected with $8 \mu\text{g}$ of pNL4.3 $\Delta\text{pol}\Delta\text{env}$
651 plasmid was collected 48h after transfection and filtered using a $0.45 \mu\text{m}$ filter. The medium was
652 then ultracentrifuged on a 20% sucrose cushion in TNE using a SW41Ti rotor (Beckman) at
653 40,000rpm during 1h30. A solution with 0.25M sucrose, 1mM EDTA, 10mM tris HCL pH 7,4
654 was used to diluted the 60% iodixanol stock solution (OptiPrep from Sigma) and to prepare a
655 40% and 20% iodixanol solution. 1.5mL of each dilution (60%, 40% and 20% iodixanol) was
656 successively layered in a SW55Ti tube (Beckman) and the pellet of VLPs obtained after
657 ultracentrifugation on a 20% sucrose cushion in TNE was loaded from the top. Tubes were
658 ultracentrifuged at 50000rpm in a SW55Ti rotor (Beckman) at 4°C for 3 hours. Then 20 fractions
659 of $200 \mu\text{L}$ were collected from the top of the tube to the bottom. $20 \mu\text{L}$ of each fraction were
660 loaded for western blotting.

661 **GUV reagents.**

662 Brain total lipid extract (131101P) and brain L- α -phosphatidylinositol-4,5-bisphosphate (PIP₂,
663 840046P) were purchased from Avanti Polar Lipids/Interchim. BODIPY-TR-C5-ceramide,
664 (BODIPY TR ceramide, D7540) and Alexa Fluor 488 C5-Maleimide (AX 488) were purchased
665 from Invitrogen. β -casein from bovine milk (>98% pure, C6905) and other reagents were
666 purchased from Sigma-Aldrich. Culture-Inserts 2 Well for self-insertion were purchased from
667 Ibidi (Silicon open chambers, 80209).

668 **Protein purification and fluorescent labelling.**

669 Recombinant mouse IRSp53 I-BAR domain was purified and labeled with AX488 dyes as
670 previously described (Prévost et al., 2015; Saarikangas et al., 2009). Recombinant HIV-1
671 immature Gag protein was purified by J. Mak as described in (Yandrapalli et al., 2016) and
672 labelled with Alexa488 maleimide dyes (Invitrogen). Briefly, a 200µM solution of the maleimide
673 dye was incubated overnight at 4°C with a 20µM solution of the Gag purified protein in a buffer
674 of pH 8.0 with 1M NaCl and 50mM Tris. Post incubation, the labelled mixture was subjected to
675 dialysis with the Slide-A-Lyzer Mini Dialysis Device (Thermo Scientific), following the
676 manufacturer's instructions to remove the excess unbound dye from the solution.

677 **GUV preparation and observation**

678 *Lipid and buffer compositions.* Lipid compositions for GUVs were brain total lipid extract (Yu
679 Seong-hyun et al., 2006) supplemented with 5 mol% brain PI(4,5)P₂. If needed, 0.5 mole%
680 BODIPY TR ceramide was present in the lipid mixture as a membrane reporter. The salt buffer
681 inside the GUVs, named I-buffer, was 50 mM NaCl, 20 mM sucrose and 20 mM Tris pH 7.5. The
682 salt buffer outside the GUVs, named O-buffer, was 60 mM NaCl and 20 mM Tris pH 7.5.

683 *GUV preparation.* GUVs were prepared by using the polyvinyl alcohol (PVA) gel-assisted
684 method (Weinberger et al., 2013a), except GUVs shown in Fig 6-Fig. S1 c in which the
685 electroformation method was used (Méléard et al., 2009). For the PVA method, a PVA solution
686 (5% (w/w) of PVA in a 280 mM sucrose solution) was warmed up to 50°C before spreading on a
687 coverslip that was cleaned in advance by being bath sonicated with 2% Hellmanex for at least 30
688 min, rinsed with MilliQ water, sonicated with 1M KOH, and finally sonicated with MilliQ water
689 for 20 min. The PVA-coated coverslip was dried in an oven at 50°C for 30 min. 5-10 µl of the
690 lipid mixture (1 mg/mL in chloroform) was spread on the PVA-coated coverslip, followed by
691 drying under vacuum for 30 min at room temperature. The PVA-lipid-coated coverslip was then
692 placed in a 10 cm cell culture dish and 0.5 mL of the inner buffer was added on the coverslip,

693 followed by keeping it stable for 45 min at room temperature to allow the GUVs to grow. For the
694 electroformation method, a few μl of lipid mixture at 3 mg/ml were deposited onto platinum
695 electrodes (Goodfellow). The lipid film was dried for at least 30 min under vacuum at room
696 temperature, and then rehydrated in I-buffer under a voltage of 0.25 V and a frequency of 500 Hz
697 overnight at 4°C (Méléard et al., 2009).

698 *Sample preparation and observation.* GUVs were first incubated with either Gag or I-BAR
699 domain at bulk concentrations depending on the designed experiments for at least 15 min at room
700 temperature before adding either I-BAR domain or Gag, respectively, into the GUV-protein
701 mixture. In experiments where there was only Gag but no I-BAR domain, the stock solution of I-
702 BAR domain was used in order to obtain a comparable salt strength outside GUVs as those where
703 I-BAR domain was present. The GUV-protein mixture was then incubated at least 15 min at room
704 temperature before observation. For the Gag/I-BAR membrane recruitment assay, samples were
705 observed on a Nikon C1 confocal microscope equipped with a X60 water immersion objective
706 (Nikon, CFI Plan Apo IR 60X WI ON 1.27 DT 0.17). For the Gag/I-BAR tubulation assay,
707 samples were observed with an inverted spinning disk confocal microscope Nikon eclipse Ti-E,
708 equipped with Yokogawa CSU-X1 confocal head, 100X CFI Plan Apo VC objective (Nikon) and
709 a CMOS camera, Prime 95B (Photometrics).

710 For all experiments, coverslips were passivated with a β -casein solution at a concentration
711 of 5 g.L⁻¹ for at least 5 min at room temperature. Experimental chambers were assembled by
712 placing a silicon open chamber on a coverslip.

713 **GUV Image analysis.**

714 Image analysis was performed by using Fiji (Schindelin et al., 2012).

715 *Quantification of AX488 Gag binding on GUV membranes.* Florescence images were taken at the
716 equatorial planes of GUVs using identical confocal microscopy settings. The background

717 intensity of the AX488 channel was obtained by manually drawing a line with a width of 10
718 pixels perpendicularly across the membrane of a GUV. We then obtained the background
719 intensity profile of the line where the x-axis of the profile is the length of the line and the y-axis is
720 the averaged pixel intensity along the width of the line. The background intensity was obtained by
721 calculating the mean value of the sum of the first 10 intensity values and the last 10 intensity
722 values of the background intensity profile. To obtain Gag fluorescence intensity on the membrane
723 of the GUV, we used membrane fluorescence signals to find the contour of the GUV (using the
724 “Fit Circle” function in Fiji). Then, a 10 pixel wide band centred on the contour of the GUV was
725 used to obtain the Gag intensity profile of the band where the x-axis of the profile is the length of
726 the band and the y-axis is the averaged pixel intensity along the width of the band. Gag
727 fluorescence intensity was then obtained by calculating the mean value of the intensity values of
728 the Gag intensity profile, following by subtracting the background intensity.

729 *Gag sorting map.* Florescence images of GUVs were taken using identical confocal microscopy
730 settings. For every GUV, we first calculated the fluorescence intensity ratio for every pixel of the
731 Gag and membrane images of a GUV using $(I_{Gag} - I_{background}^{Gag})/I_{membrane}$, where I_{Gag} is the
732 Gag intensity, $I_{background}^{Gag}$ is the background intensity in the Gag channel, and $I_{membrane}$ is the
733 membrane intensity. The sorting map was then obtained by converting the resulting image from
734 the previous step to a pseudo-colored image via the “Look Up Table, Phase” in Fiji. The
735 background intensity value in the Gag channel was the man intensity value of a 50 pixel wide
736 square in the background outside GUVs. The sorting map of I-BAR domain was obtained by
737 using the same procedure as those for Gag.

738 **Statistics.**

739 All notched boxes show the median (central line), the 25th and 75th percentiles (the bottom and
740 top edges of the box), the most extreme data points the algorithm considers to be not outliers (the
741 whiskers), and the outliers (crosses).

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Figures

Figure 1: Partial knockdown of IRSp53 decreases HIV-1 Gag particle release by arresting assembly at the cell plasma membrane. a) siRNA knockdown of IRSp53 expression in Jurkat T lymphocytes leads to a significant decrease in pNL4-3ΔEnv Gag particle release (see graph and immunoblots for IRSp53, $p=0.00265$, Student's t-test, and loading controls beneath the graph). b) Similarly, knockdown of IRSp53 expression in Gag expressing HEK293T cells led to a significant decrease in HIV-1 Gag particle release ($p=0.00487$, Student's t-test) as compared to siRNA IRTKS ($p=0.0116$, Student's t-test). On the other

hand, knockdown of IRTKS expression (a closely related I-BAR protein) did not have a significant effect on particle release ($p=0.0924$, Student's t-test, upper graph, immunoblots for IRSp53, IRTKS and loading controls beneath the graph) ($n=3$ independent experiments). Another multiple comparison statistical test ANOVA was applied to compare the 3 siRNA conditions showing a significative difference with a p value equal to 0.0089. **c)** Transmission electron microscopy images of HEK293T cells expressing HIV-1 Gag with siRNA control (upper panel) and siRNA IRSp53 (lower panel). Scale bar is $0.5\mu\text{m}$ (upper image) and is $1\mu\text{m}$ (lower image). **d)** Transmission electron microscopy zoomed images of viral buds from HIV-1 Gag expressing cells treated with siRNA-mediated knocked down of IRSp53 (lower panel) showing arrested buds at the plasma membrane as compared to the siRNA control cells (upper panel) which display a normal range of buds in different stages of assembly and budding (scale bar is 100 nm). **e)** Measurement of the bud dimensions (height and width median with interquartile) in the control siRNA and siRNA IRSp53 conditions ($n=145$ buds from 14 different cells for each condition, $N=2$ independent experiments). The knocked down cells exhibit a narrow range of heights corresponding to the arrested buds visible in the images while the control cells display a wider range of heights corresponding to assembly progression (left graph, "Height of bud"). Distribution of the height values in the two conditions are significantly different ($p=1.05 \times 10^{-28}$, Kolmogorov-Smirnov test). On the opposite, the widths of the buds in both conditions did not display significant differences in distributions. ($p=0.0609$, Kolmogorov-Smirnov test).

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958 **Figure 2: Intracellular HIV-1 Gag and IRSp53 complexation and cell membrane binding.**

959 **a)** Co-immunoprecipitation of HIV-1 Gag/IRSp53 with an anti-IRSp53 antibody. HIV-1 Gag is
960 enriched in the anti-IRSp53 pulldown (lane 1) as compared to the controls (lane 2: IP with an
961 anti-Rabbit serum; lane 3: no antibody; lane 4: Mock, i.e. without Gag). **b)** Co-
962 Immunoprecipitation of HIV-1 Gag/ectopic IRSp53-GFP with an anti-GFP antibody upon over-
963 expression of HIV-1 Gag and ectopic IRSp53-GFP in transfected HEK293 T cells. HIV-1 Gag is
964 enriched in the anti-IRSp53-GFP pulldown (lane 3: transfected HEK293T cell lysate containing
965 IRSp53-GFP and HIV-1 Gag), as compared to the controls (lane 1: Mock without Gag or IRSp53-
966 GFP; lane 2: IRSp53 alone; lane 4: Gag alone). Input, IP anti-GFP and Flowthrough after IP are
967 shown. **c)** Membrane flotation assay protocol: (1) 293T cells were dounced, (2) the post-nuclear
968 supernatant was loaded on a discontinuous sucrose gradient, and (3) following ultracentrifugation,
969 cell membranes (lysosomal associated membrane protein, Lamp2 biomarker, fractions 1-3) were
970 separated from the cytosolic fraction (ribosomal S6 protein biomarker, fractions 6-8). **d)**
971 Immunoblots of the indicated proteins (on the left) and quantification of the % of protein
972 membrane binding in the graph below show that upon HIV-1 Gag expression in cells, IRSp53 is
973 significantly enriched by 2-fold in the cell membrane fraction (p value=0.000129; ***, Student's

974 t-test) (n=5 independent experiments). A similar increase is observed for Tsg-101, a known
975 interactor of the p6 domain of Gag (p value=0.00517; **).

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HEK 293T

Jurkat T cells

Figure 3: Super resolution microscopy imaging reveal preferential IRSp53 localization at HIV-1 Gag budding sites. a,b- Super-resolved PALM/STORM dual color images of

HEK293T cells expressing Gag(i)mEos2 (green) and immuno-labelled for IRSp53 (red, **a**) or IRTKS (red, **b**) (scale bar 10 μ m) with a magnified view (scale bar 500 nm) and selected zoom-in images of single Gag(i)mEos2 clusters (scale bar 100nm). **c,d**- Quantification of coordinate based colocalization (as in (Malkusch et al., 2012)) at Gag assembly sites: CBC values for IRSp53 (**c**) and IRTKS (**d**) were plotted as relative frequencies. IRSp53 CBC distribution (**c**) shows that 27% of all IRSp53 localizations are highly correlated with Gag(i)mEos2 localizations (>0.5). On the other hand, only 14% of IRTKS localizations (**d**) are highly correlated, while IRTKS CBC distribution exhibits a peak of anti-correlated/non correlated (-0.5 to 0) localization. **e,f**, Super resolved dual color PALM/STORM images of Jurkat T cells expressing Gag(i)mEos2 (green) and immuno-labelled for IRSp53 (red, **e**) or IRTKS (red, **f**) with a magnified view (scale bar 500 nm) and single Gag(i)mEos2 cluster zoom (scale bar 100nm). **g,h**- Relative frequency distribution plot of the CBC values for IRSp53 (**g**) and IRTKS (**h**). **g** shows that 26% of IRSp53 localizations are highly correlated with Gag(i)mEos2 localizations (>0.5) while **h** shows that IRTKS localisations are mainly anti-correlated to non-correlated (-0.5 to 0) with Gag(i)mEos2 localizations.

Figure 4: Numerical simulations confirm preferential IRSp53 localization around HIV-1 Gag budding sites.

a- Schematic representation of the different configurations used in the numerical simulation mimicking belts of given waists (w) of IRSp53 or IRTKS localizations (red) surrounding an HIV-1Gag(i)mEos2 assembly site (green) at a given distance (d). **b-** Numerically simulated cumulative distribution function of CBC values obtained for a 20 nm waist belt of IRSp53 or IRTKS at different distance from 0 to 160 nm (top). Below are found the schematic representations (upper part, input) and the Thunderstorm reconstructed images obtained from the simulated positions (lower part, output, see method for details) for the 20 nm waist belt at different positions. **c,d,e,f** - Experimental CBC values for IRSp53 in HEK (**c**) or Jurkat T-cells (**g**) and IRTKS in HEK (**d**) and Jurkat T-cells (**f**) in were plotted as cumulative frequency distributions and compared to simulated distributions obtained at different distances and structures (see FigS9 for the whole data sets). For both cell type, IRSp53 shows a cumulative CBC distribution corresponding to a simulation with a waist of 40nm (width 80 nm) at a distance of 80nm (left graph, bold grey lines correspond to the experimental data for IRSp53, bold blue lines correspond to the simulated values closest to experimental data, see FigS9 for statistics). IRSp53 thus corresponds to a restricted pattern in and around a Gag assembly site (panel 1 schematic of simulated data, panel 2 simulated data and panel 3 experimental data). On the other hand, the IRTKS experimental CBC distribution (bold grey line in the graph) is similar to simulations with a waist of 100nm (width 200nm) at a distance of 140nm (bold red line). IRTKS is more diffuse and spreads out (panel 1 schematic of simulated data, panel 2 simulated data and panel 3 experimental data). Scale bar in the panels = 100nm.

Figure 5: IRSp53 is incorporated into HIV-1 particles in a Gag-dependent manner. a)

Schematic for the protocol followed for imaging and analysis. HIV-1 Gag VLPs were purified from HEK293T cells expressing HIV-1 Gag/Gag-mCherry and IRSp53-GFP or other GFP tagged proteins binding PI(4,5)P2 (IRSp53-IBAR-GFP, IRTKS-GFP and PH-

PLC δ -GFP). Purified Gag VLPs were then spotted over a poly-lysine treated glass slide and imaged by TIRF-Microscopy (particles were imaged in the red, and IRSp53 in the green channel). For each condition, 3000 particles (~300 particles/image, 10 images) were counted. Fluorescence correlation (Mander's coefficient, see Materials and Methods for details) was determined for Gag-mCherry and for IRSp53-GFP, IRTKS-GFP and PH-PLC δ -GFP and reported in the graphs. b) The 0.5 value indicates a random incorporation level (indicated by black line across the graph). IRSp53-GFP and IRSp53-IBAR show high correlation values (0.95-1 and 0.8, respectively). The other I-BAR domain proteins were not significantly correlated with Gag-mCherry particles (0.4-0.5). PH-PLC δ -GFP, a known marker of the phospholipid PI(4,5)P₂, shows a slightly higher correlation (0.6), since HIV-1 Gag is known to associate with this phospholipid. c) Incorporation of IRSp53 into wild type pNL4-3 HIV-1 or d) Gag VLPs revealed by immunoblots against Gag(p24), IRSp53, IRTKS, Tsg101 or actin, as indicated. Following a 25% sucrose cushion purification, IRSp53 was found to be associated with released wild type HIV-1 (left panel) and Gag VLPs (right panel). Tsg101, known to be incorporated into released particles, was also found associated with viral particles. IRTKS, a closely related I-BAR protein to IRSp53, was not incorporated in purified HIV-1 viral particles or Gag-VLPs. e) Protocol of VLPs purification using sucrose cushions and an iodixanol gradient. Briefly, pellets obtained after ultracentrifugation of cell culture medium of HEK293T transfected with pNL4.3HIV-1 or pGag were deposited on an iodixanol gradient (20, 30 and 60%). 20 fractions of 200 μ L were collected from the top of the tube. Fractions collected following an iodixanol gradient purification of NL4-3 Δ Pol Δ Env Gag VLPs were analyzed using western blots for IRSp53 and Gag, TSG101 and ALIX, CD81 and CD63 revealed respectively on the same membrane (blot 1 and blot 2) revealing IRSp53 association with Gag viral particles and known cofactors.

Figure 6: IRSp53 I-BAR domain enhances Gag recruitment to GUV-membranes and at the tip of I-BAR domain-induced tubes. a) (Left) AX488 Gag fluorescence intensity on membranes in the absence of I-BAR domain (named “Gag only”), in the presence of I-BAR domain where

1059 GUVs were first incubated with I-BAR domain and then Gag (named “I-BAR + Gag”) and GUVs
 1060 were first incubated with Gag and then I-BAR domain (named “Gag + I-BAR”). Each circle
 1061 presents one GUV analysis. N = 82 GUVs, n = 4 sample preparations for “Gag only”, N = 67
 1062 GUVs, n = 4 sample preparations for “I-BAR + Gag”, and N = 104 GUVs, n = 4 sample
 1063 preparations for “Gag + I-BAR”. To pool all data points from the 4 sample preparations, in each
 1064 preparation for all three conditions, Gag intensities were normalized by the mean Gag intensity in
 1065 the “Gag only” condition. Protein bulk concentrations: 0.3 μ M for AX488 Gag and 0.5 μ M for I-
 1066 BAR domain (not fluorescently labelled). (Right) Representative confocal images of AX488 Gag
 1067 on GUV membranes in “I-BAR + Gag” condition. To visualize GUV membranes, 0.5 mole% of
 1068 BODIPY-TR-C5-ceramide was incorporated in the membranes. b) Representative confocal
 1069 images of AX594 Gag in I-BAR domain-induced tubules. Inverted grayscale images are shown
 1070 for I-BAR domain and Gag. Protein bulk concentrations: 0.3 μ M for AX594 Gag and 0.05 μ M for
 1071 I-BAR domain (70% unlabeled and 30% AX488 labeled I-BAR domain). Cyan arrowheads point
 1072 out I-BAR domain-induced tubules and white arrows indicate the co-localization of Gag and I-
 1073 BAR domain at the tips of the tubules. c and d) Representative confocal images of AX488 Gag
 1074 (green) in I-BAR domain-induced tubules. Protein bulk concentrations: in (c) 0.1 μ M for AX488
 1075 Gag and 0.05 μ M for I-BAR domain (not fluorescently labelled); in (d) 0.3 μ M for AX488 Gag
 1076 and 0.05 μ M for I-BAR domain (not fluorescently labelled). To visualize GUV membranes, 0.5
 1077 mole% of BODIPY-TR-C5-ceramide (magenta) was incorporated in the membranes. In (c),
 1078 inverted grayscale images were shown for membranes and Gag. The cyan arrowhead points out
 1079 an I-BAR domain-induced tubule and white arrow indicate Gag signals at the tip of the tubule.
 1080 Sorting map was obtained by calculating the fluorescence intensity ratio of Gag and membranes
 1081 (see Material and Methods for more details). In (d), dashed white lines indicate I-BAR domain
 1082 induced tubules. Scale bars, (a - c) 5 μ m and (d) 2 μ m.

1083

1084 **Supplemental figure legends**

1085 **Figure 1-figure supplement 1: Effect of siRNA-based knockdown of IRSp53 and**
1086 **IRTKS gene expression on HIV-1 Gag particle release.** a) Expression of HIV-1 Gag in
1087 HEK293T cells (from molecular clones pNL43ΔPolΔEnv or pGag – optimized HIV-1 Gag) and a
1088 schematic representation of the viral Gag polyprotein. The HIV-1 Gag Pr55 polyprotein is known
1089 to have four main domains, the matrix MA interacting with cell membrane thanks to its lipid
1090 binding site and myristate (myr), the capsid CA for Gag-Gag oligomerization, the nucleocapsid
1091 NC interacting with the viral genomic RNA and p6 recruiting Tsg101 (ESCRT protein) for
1092 particle budding. b) Validation of siRNA targeting IRSp53 gene expression. SiRNA targeting
1093 IRSp53 diminishes the expression of IRSp53-GFP as seen by immunoblots (Left panel, Lane 1:
1094 siCTRL; Lane 2: siIRSp53) and fluorescence imaging of transfected HEK293T cells expressing
1095 ectopic IRSp53-GFP with the corresponding siRNA (right panel). c) Validation of siRNA
1096 targeting ectopic IRTKS. siRNA targeting IRTKS diminishes expression of IRTKS-GFP as seen
1097 by immunoblots (Left, Lane 1: siCTRL; Lane 3: siIRTKS) and fluorescence imaging of
1098 transfected HEK293T cells expressing IRTKS-GFP with siRNA, as indicated (right panel). d)
1099 Reduced viral release are seen in cells knockdown for endogenous IRSp53 expression
1100 (representative immunoblots, Lane 1: siCTRL; Lane 2: siIRSp53). e) Endogenous IRTKS
1101 knockdown has no significant effect on HIV-1 Gag particle release (representative immunoblots,
1102 Lane 1: siCTRL; Lane 3: siIRTKS). Arrows (<) show the corresponding proteins of interest.

1103 **Figure 1-figure supplement 2: Protein sequence comparison of IRSp53 and IRTKS.** a)
1104 IRSp53 and IRTKS are membrane curving I-BAR proteins. b) IRSp53 (Query, Accession
1105 Q9QB8) shares a 40% sequence homology with IRTKS (Subject, Accession Q9UHR4) Sequence
1106 alignment performed with NCBI Protein BLAST. Most of this homology is centered in the I-
1107 BAR/IMD domain of the three proteins and the C terminal SRC Homology 3 (SH3) domain
1108 common to the two proteins.

1109 **Figure 1-figure supplement 3: Transmission electron microscopy of siRNA treated**
1110 **HEK293T cells expressing HIV-1 Gag.** SiRNA control (a) or siRNA IRSp53 (b) treated cells
1111 reveal that IRSp53 protein depletion in cells display HIV-1 Gag arrested assembly at the cell
1112 plasma membrane as shown by transmission electron microscopy. Scale bar is 0,5 μm or 1 μm as
1113 indicating in the images.

1114 **Figure 2-figure supplement 1: Complexation of IRSp53 with Gag(i)mEOS2 is independent**
1115 **of Gag-p6 domain.** Addition of an internal mEos2 tag within the Gag protein does not affect its
1116 complexation with IRSp53 (Compare Lane 1 and Lane 2). Gag(i)mEos2 Δ p6, a mutant of Gag
1117 deficient in p6 dependent-ESCRT recruitment, is also immuno-precipitated (IP) with an IRSp53
1118 antibody (Lane 3), indicating that the loss of p6, and most probably most of Tsg101 recruitment,
1119 is not a limiting factor for Gag-IRSp53 complexation. Lane 4 is an IP control (Mock = no Gag).

1120 **Figure 3-figure supplement 1: Localisation precision and size determination of HIV-Gag**
1121 **Clusters.**

1122 **a)** Distribution of localisation precisions for PALM (in green) or STORM (in red) as given by
1123 Thunderstorm analysis in Fiji. Localisation precision distribution exhibit maxima at 16 nm for
1124 PALM, corresponding to a mean value of 20 ± 5 nm and 26 nm for STORM. The localization
1125 precision is obtained by eq 17 of (1). **b)** Calibration of clusters size using HIV-1 Gag(i)m-Eos2
1126 virus like particles. *Left* : Superimposition of classical TIRF image (in red) and its PALM image
1127 equivalence (in green) for HIV-1 Gag(i)m-Eos2 virus like particles (scale bar=500nm). The inset
1128 is a zoom of a VLP (scale bar = 100 nm) - *Middle* : PALM image normalized pixel intensity as a
1129 function of the distance from the centre of the VLP (blue dots). Red is a Gaussian fit of this pixel
1130 intensity. The size of the particle is estimated as the waist of the Gaussian at 10% of the
1131 maximum as in (2). *Right* : Comparison of VLP size distribution obtained by Voronoi based
1132 segmentation and DBSCAN clusterization methods using SR-Tesseler (3). The two methods are

1133 parametrized to identify all the clusters containing more than 50 localizations in the different VLP
1134 images (n=7). DBSCAN exhibit a more relevant median value (med=114 nm) than Voronoi based
1135 segmentation clusters distribution (med=90 nm) does. Moreover from the DBSCAN distribution
1136 it can be seen a maxima at 120-130 nm, corresponding to the VLP size obtained by Gaussian
1137 fitting as in (2). **c,d,e,f**) DBSCAN size distribution of the HIV-1 Gag(i)-mEos2 clusters found in
1138 the different cells studied here (HEK, total n=10 cells, n=5 for IRSp53 and n=5 for IRTKS, Jurkat
1139 T-cells, total n= 9 cells, n=5 for IRSp53, n=4 for IRTKS). All the distribution exhibit median
1140 values around 100 nm (see main text for exact values with IQR error), below or equivalent to the
1141 size of VLP.

1142 **Figure 3-figure supplement 2: Super resolution PALM/STORM images of HEK293T cells**
1143 **expressing HIV-1 Gag(i)mEos2** (in green) and immunolabelled for **a)** IRSp53 (Atto647N, in
1144 red) and **b)** IRTKS (Atto647N, in red), with magnified images adjacent to the main image. Scale
1145 bar is 10µm for the large images and 500 nm for the zoomed-in images.

1146 **Figure 3-figure supplement 3: Super resolution PALM/STORM images of Jurkat T cells**
1147 **expressing HIV-1 Gag(i)mEos2** (in green) and immunolabelled for **a)** IRSp53 (Atto647N, in
1148 red) and **b)** IRTKS (Atto647N, red), with magnified images adjacent to the main image. Scale bar
1149 is 10µm for the large images and 500 nm for the zoomed-in images.

1150 **Figure 3-figure supplement 4: Workflow of image analysis for PALM/STORM images.** (1)
1151 Reconstructed PALM images (2) The PALM images were thresholded in ImageJ to obtain a mask
1152 of the assembly sites of Gag. (3) The coordinates of centres of these assembly sites were then
1153 obtained using the Analyze Particles feature of ImageJ. (4) These coordinates were then used to
1154 analyze the original PALM and STORM single molecule localizations, and extract PALM
1155 coordinates from an area corresponding to size of assembly sites ($r < 80\text{nm}$ from centre), and
1156 STORM coordinates in a larger area around the assembly site ($r < 150\text{nm}$ from centre). (5) Using
1157 these filters, a new set of PALM and STORM localizations were generated, which corresponded

to Gag assembly sites and the area around these sites. (6) These localizations were then used to calculate the coordinate based colocalization (CBC) for PALM (Gag) and STORM (IRSp53/IRTKS). The CBC values were then plotted as cumulative probability distributions.

Figure 4-figure supplement 1: Cumulative frequency distributions of CBCs for simulated PALM/STORM data. Simulations corresponding to different patterns of STORM localizations around PALM clusters were performed using a homemade macro in Fiji. a) Each molecule position was randomly distributed within a fixed size disk of 100nm diameter for the PALM localizations (which represent 70-80% of all the experimental Gag clusters size found) or within a belt surrounding this disk for the STORM localization. The STORM belt size ranged from a waist of 20 nm (width 40nm) to a waist of 100 nm (width 200nm), with distances from centre of the simulated viral bud (PALM disk) to the centre of the surrounding belt ranging from 0 nm to 160 nm for each waist size. Each simulated localization was then convoluted by a 2D spatial Gaussian function with a waist of 200nm in x and y direction to simulate the point spread function of the microscope. The number of photons emitted by each simulated position was randomly distributed according a Gaussian distribution centered to a value equivalent to the maxima of the distribution of the one experimentally observed, this in order to obtain a localization precision equivalent to the experimental ones (see Fig S5 for the experimental localization precision). Each set of simulated images was then analyzed using the Thunderstorm plugin of Fiji with the same parameter as the one used for experimental data. Single molecule localizations obtained from this analysis were then used for CBC analysis using the CBC plugin of Thunderstorm as it was done for experimental data sets (b to f). (g,h, i, j) Map of the root mean square error obtained when comparing the experimental cumulative cbc distribution to the simulated cbc distributions for the different conditions tested here. The bluest dot of the map corresponds to the minimal value of the RMSE test, i.e. the best fit. These minimal values (red circles) correspond to a belt of 40nm waist centered at a distance of 80 nm from the HIV-1 Gag cluster centre in the case of IRSp53 and to a

1183 belt of 100 nm waist centered at a distance of 140 nm from the HIV-1 Gag cluster centre in the
1184 case of IRTKS, this for both cell types. Direct comparison of the whole RMSE maps obtained for
1185 the 2 conditions independently of the cell type shows that on average, IRTKS molecules are
1186 located further of the HIV-1 Gag(i)mEos clusters than IRSp53 molecules are.

1187 **Figure 6- figure supplemental 1: a,b) In vitro GUV experiments showing an increase of**
1188 **IRSp53 I-BAR binding on GUV upon HIV-1 Gag addition.** (a) Representative confocal
1189 images of AX488 labelled IRSp53 I-BAR and AX546 labelled Gag on GUV membranes in “I-
1190 BAR + Gag” condition. (b) Measurement of AX488 IRSp53 I-BAR fluorescence intensity on
1191 membranes in the absence of Gag (named “I-BAR only”), in the presence of Gag where GUVs
1192 were first incubated with IRSp53 I-BAR domain and then with HIV-1 Gag (named “I-BAR +
1193 Gag”). Each circle presents one GUV analysis. N = 27 GUVs, n = 2 sample preparations for “I-
1194 BAR only”, N = 32 GUVs, n = 2 sample preparations for “I-BAR + Gag”. To pool all data points
1195 from the 2 sample preparations, I-BAR intensities were normalized by the mean I-BAR intensity
1196 in the “I-BAR only” condition. Protein bulk concentrations: 0.05 μ M for I-BAR domain and 0.3
1197 μ M for Gag. $**p = 0.01133$, Student’s *t*-test. (c, d) **In vitro GUV experiments.** (c)
1198 Representative confocal images of a GUV (the membrane contains 0.5 mole% of BODIPY-TR-
1199 C5-ceramide, red) incubating with a low concentration of IRSp53-I-BAR domain (0.05 μ M,
1200 composed of 70% of unlabeled I-BAR domain and 30% of AX488 labelled I-BAR domain,
1201 green). The I-BAR domain induces membrane tubulation towards the interior of the GUV. (d)
1202 Representative confocal images of a GUV first incubated with IRSp53 I-BAR domain (0.05 μ M,
1203 unlabelled), followed by addition of HIV-1 Gag (0.3 μ M, AX488 labelled, green). GUV
1204 membranes contained 0.5 mole% of BODIPY-TR-C5-ceramide (red). The white arrow points to
1205 the accumulation of AX488 Gag singles at the tip of an I-BAR induced tube. Scale bars, 5 μ m.

1206 **Figure 6- figure supplemental 2: Dotty signals of Gag in GUVs with I-BAR induced tubules.**
1207 Representative confocal images of GUVs first incubated with IRSp53 I-BAR domain (0.05 μ M,

unlabelled), followed by addition of HIV-1 Gag (0.3 μ M, AX488 labelled, green). GUV membranes contained 0.5 mole% of BODIPY-TR-C5-ceramide (magenta). White arrows point to the accumulation of AX488 Gag singles at the tip of I-BAR induced tubes. Scale bars, 5 μ m.

1211

1212 **Video legends**

1213 **Video 1. Imaging of a I-BAR domain induced tubule having Gag signal at its tip.**

1214 Time-lapse imaging of a I-BAR domain induced tubule. GUVs were first incubated with IRSp53
1215 I-BAR domain (0.05 μ M, unlabelled), followed by addition of HIV-1 Gag (0.3 μ M, AX488
1216 labelled, green). GUV membranes contained 0.5 mole% of BODIPY-TR-C5-ceramide (magenta).
1217 Frame interval, 0.6 sec. Time in mm:ss. Scale bar, 2 μ m.

1218 **Video 2. Imaging of a GUV having I-BAR domain induced tubules.**

1219 GUVs were first incubated with IRSp53 I-BAR domain (0.05 μ M, unlabelled), followed by
1220 addition of HIV-1 Gag (0.3 μ M, AX488 labelled, green). GUV membranes contained 0.5 mole%
1221 of BODIPY-TR-C5-ceramide (magenta). Frame interval, 0.6 sec. Time in mm:ss. Scale bar, 5
1222 μ m.

1223 **Video 3. Imaging of a GUV having I-BAR domain induced tubules.**

1224 GUVs were first incubated with IRSp53 I-BAR domain (0.05 μ M, unlabelled), followed by
1225 addition of HIV-1 Gag (0.3 μ M, AX488 labelled, green). GUV membranes contained 0.5 mole%
1226 of BODIPY-TR-C5-ceramide (magenta). Frame interval, 0.6 sec. Time in mm:ss. Scale bar, 5
1227 μ m.

1228

1229 **Source data files**

1230 **Figure 1 - source data 1:** Immunoblot Quantification using Fiji for Figure 1B graph

1231 **Figure 1- source data 2:** Heights and Widths of HIV-1 Gag bud measurements for Figure 1E
1232 graph
1233 **Figure 2 - source data 1:** Membrane flotation Quantification using Fiji for Figure 2D graph
1234 **Figure 3- source data 1:** Experimental data CBC for IRSp53
1235 **Figure 3- source data 2:** Experimental data CBC for IRTKS
1236 **Figure 3- source data 3:** cluster sizes for IRSp53
1237 **Figure 3- source data 4:** cluster sizes for IRTKS
1238 **Figure 4- source data 1:** simulation-CBC-dataset-1 for IRSp53
1239 **Figure 4- source data 2:** simulation-CBC-dataset-2 for IRTKS
1240
1241 **Supplementary File 1: Key Resources Table 1**

a **b**

IRTKS-IRSp53 proteins sequences alignment

Score	Expect	Method	Identities	Positives	Gaps
396 bits(1017)	7e-137	Compositional matrix adjust.	216/539(40%)	320/539(59%)	56/539(10%)
Query 7	EEMHRLTENYKTIMEQFNPSLRNFIAMGKNYEKALAGVTYAAKG YFDALVKMGELASES	66			
Sbjct 6	EE++RLTE+ Y+ +MEQFNP LRN I +GKNYEKA+ + A K Y+D + K+GE+A+ S	65			
Query 67	QGSKELG DVL FQMAEVHRQIQNQLEEMLKSFHNELLTQLEQKVELDSRYLSAALKKYQTE	126			
Sbjct 66	EEVNRLTESTYRNVMEQFNPLRLNLINLGKNYEKAVNAMILAGKAYDGVAKIGEIA TGS	125			
Query 127	QRSGDALDKCQAE LKKLRKKSQGSKNPQKYSDELQYIDAISNKQGELENYVSDGYKTA	186			
Sbjct 126	++K ++L+K QAE LKK+R+KSQGS+N KY KE++Y++ ++++Q E++ +++DG K A	185			
Query 187	HKNKLESLEKSQAE LKKIRRKSGSRNALKYEHKEIEYVETVTSRQSEIQKF IADGCKEA	246			
Sbjct 186	LTEERRRFCFLVEKQCAVAKNSAAYHSGKELLAQKLPLWQQACADPSKIPERAVQLMQQ	245			
Query 247	L EE+RRFCFLV+K C A + YH + ELL KLP WQ+ C D K+PE+ + ++++	306			
Sbjct 246	LLEEKRRFCFLVDRKHC GFANHIHYHLSAELLNSKLPRWQETCVD AIKVPEKIMNMIEE	271			
Query 307	VASNGATLPSALSASKSNLVISDPIPGAKPLFPVPELAPFVGRMSAQESTPIMNGVTGPD	363			
Sbjct 272	+ + P + P+ P+ +P + R +	328			
Query 364	IKT-----PASTPVSGTPQASPMIERSNVVR-----	423			
Query 307	GEDYSPWADRKA AQP KSLSP PQSQSKLSDSYSN---TLPVRKSVTPKNSYATTENKTLPR	363			
Sbjct 272	+DY + P + S S L D ++N P + V NS T+E+ +L R	328			
Query 364	-KDYDTLSKCS PKMPPAPSGRAYTSPLIDMFNNPATAAPNSQRVN--NSTGTSEDPSLQR	423			
Sbjct 329	SSSMAAGLERNGRMRVKAIFSHAAGDNSTLLSFKEGDLITLLVPEARDGWHYGESEKTKM	388			
Query 424	S S+A GL + +VK IF H AG N TLLSF +GD+ITLL+PE +DGW YGE + +K	472			
Sbjct 389	SVSVATGLNMMKKQKVKTIFPHTAGSNKTLLSFAQGDVITLLIPEEKDGWLYGEHDVSKA	448			
Query 473	RGWFPPFSYTRVLDS DGS DRLHM---SLQQGKSSSTGNLLDKDDLAI PPPDY-----G	528			
Sbjct 449	RGWFP SYT++L+ + ++ + + S +S ST NL + + IPPPDY	505			
Query 473	RGWFPSYTKLLEENETEAVTVPTPSPTFVRSISTVNLSSENSSVVIPPDPYLECLSMGAA	528			
Sbjct 449	AASRAFP AQTASGFK---QRPYSVAVPAFSQGLDDYGARMSRNPF AHVQLKPTVTNDR	505			
Query 473	A RA A+T S FK +P A P + G S NPFA V+L+PTVTNDR	528			
Sbjct 449	ADRRADSARTTSTFKAPASKP-ETAAPNDANGTAKPPFLS-GENPFATVKLRPTVTNDR	505			

a

HIV-1 Gag + siCTRL

b

HIV-1 Gag + siIRSp53

IP α IRSp53

Input

Anti-CAP24

GAPDH

1. Gag
2. Gag(i)mEOS2
3. Gag(i)mEOS2 Δ p6
4. MOCK

b

a **b**

a **b**

Thunderstorm
reconstructed
PALM image

①

②

③
matrix of x,y
coordinates
of assembly
sites centers

MATLAB

④

⑥
CBC
analysis

⑤

Filtered
PALM

Filtered
STORM

MATLAB

Assembly sites
segmented image

- PALM Localisations (Gag)
- STORM Localisations (IRSp53/IRTKS)

Membrane

Gag