KIF16B mediates anterograde transport and modulates lysosomal degradation of the HIV-1 envelope glycoprotein

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ABSTRACT

The human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein (Env) is incorporated into virions at the site of particle assembly on the plasma membrane (PM). The route taken by Env to reach the site of assembly and particle incorporation remains incompletely understood. Following initial delivery to the PM through the secretory pathway, Env is rapidly endocytosed, suggesting that recycling is required for particle incorporation. Endosomes marked by the small GTPase Rab14 have been previously shown to play a role in Env trafficking. Here we examined the role of KIF16B, the molecular motor protein that directs outward movement of Rab14-dependent cargo, in Env trafficking. Env colocalized extensively with KIF16B+ endosomes at the cellular periphery, while expression of a motor-deficient mutant of KIF16B redistributed Env to a perinuclear location. The half-life of Env labeled at the cell surface was markedly reduced in the absence of KIF16B, while a normal half-life was restored through inhibition of lysosomal degradation. In the absence of KIF16B, Env expression on the surface of cells was reduced, leading to a reduction in Env incorporation into particles and a corresponding reduction in particle infectivity. HIV-1 replication in KIF16B knockout cells was substantially reduced as compared to wildtype cells. These results indicate that KIF16B regulates an outward sorting step involved in Env trafficking, thereby limiting lysosomal degradation and enhancing particle incorporation.

IMPORTANCE The HIV-1 envelope glycoprotein is an essential component of HIV-1 particles. The cellular pathways that contribute to incorporation of envelope into particles are not fully understood. Here we identify KIF16B, a motor protein that directs movement

40 from internal compartments toward the plasma membrane, as a host factor that prevents
41 envelope degradation and enhances particle incorporation. This is the first host motor
42 protein identified that contributes to HIV-1 envelope incorporation and replication.

43

44 **INTRODUCTION**

45 The HIV-1 Env protein plays critical roles in the viral lifecycle, mediating receptor and
46 coreceptor binding, fusion, and entry into target cells. Env is the principal neutralizing
47 determinant on the surface of virions, and has been a primary focus of vaccine design
48 efforts since the onset of the HIV pandemic. While many aspects of Env structure and
49 function are now understood, the determinants of particle incorporation of Env are not
50 fully defined. HIV-1 Env is synthesized on endoplasmic reticulum (ER)-bound ribosomes
51 as a precursor gp160 protein. Trimerization and initial glycosylation of Env take place in
52 the ER, followed by additional glycan modifications and cleavage of gp160 into gp120
53 (SU) and gp41 (TM) subunits in the Golgi apparatus. After delivery to the PM, the
54 heterotrimeric Env complex is rapidly endocytosed into early endosomes (1-3). The
55 trafficking events that occur following Env endocytosis are poorly defined, and are the
56 subject of this report.

57 Clues to Env trafficking and particle incorporation have come from studies of the Env
58 cytoplasmic tail (CT). Long CTs are a characteristic feature of most lentiviral Env proteins.
59 HIV-1, HIV-2, and simian immunodeficiency virus (SIV) have Env CTs of approximately
60 150 amino acids. The CT of HIV-1 Env contains multiple functional motifs that have been
61 implicated in intracellular trafficking events [reviewed in (4, 5)], suggesting that the CT
62 interacts with host trafficking molecules to direct intracellular trafficking and particle
63 incorporation of Env. In support of the critical role of the CT, Murakami and Freed showed
64 that there are remarkable cell type-specific differences in incorporation of Env with a large
65 deletion in the CT (6). While some cell lines such as MT-4 and 293T cells readily
66 incorporate Env bearing a truncated CT, incorporation into virions in most T cell lines, in

67 primary T lymphocytes, and in monocyte-derived macrophages (MDMs) requires an intact
68 CT (6-9). HeLa cells demonstrate an intermediate phenotype, with a reduction but not
69 complete loss of Env incorporation with a CT truncation (6). The cell type-dependent
70 incorporation of Env with a truncated CT, together with evidence supporting trafficking
71 motifs within the CT, suggests that specific host factors are required for incorporation of
72 Env into particles.

73 We previously demonstrated that a Rab-related adaptor protein Rab11-FIP1C
74 (FIP1C) plays a role in Env incorporation, and outlined a key role for recycling pathways
75 in this process (10-12). The specific Rab GTPase involved in FIP1C-mediated recycling
76 of Env was shown to be Rab14 (10). Additional evidence supporting a role for Rab14
77 comes from Hoffman and colleagues, who demonstrated that Env traffics to Rab14+ late
78 endosomes and lysosomes (13). Recycling of Env to the PM was demonstrated in this
79 study, and a potential role for Rab14 in regulating lysosomal degradation vs. delivery to
80 the PM was suggested. Given the accumulating evidence for Rab14 in the intracellular
81 trafficking of Env, we sought to evaluate the role of the molecular motor protein that
82 associates with Rab14, KIF16B.

83 KIF16B is a member of the kinesin-3 family of molecular motor proteins. KIF16B is a
84 plus end-directed molecular motor that regulates the steady-state distribution of Rab5+
85 early endosomes and plays a role in recycling of endocytosed cargo to the cell surface
86 (14). A seminal study describing KIF16B function demonstrated that disruption of KIF16B
87 led to redistribution of epidermal growth factor receptor (EGFR) and enhanced its
88 degradation in the lysosome (14). KIF16B has also been implicated in anterograde
89 transport of transferrin receptor and fibroblast growth factor receptor (FGFR), in

90 contributing to MHC class I cross-presentation, and in directing apical transcytosis of
91 cargo in epithelial cells (14-17). KIF16B binds directly to Rab14, and a Rab14-KIF16B
92 complex has been shown to be essential for transport of FGFR and in mediating antigen
93 cross-presentation by dendritic cells (15, 16). Given the role of Rab14 in HIV-1 Env
94 trafficking, we hypothesized that KIF16B-mediated transport plays a role in anterograde
95 trafficking of Env. We found that KIF16B directs Env to early endosomes located at the
96 periphery of cells, while expression of a motor-deficient KIF16B molecule retained Env in
97 perinuclear endosomal compartments. Measurement of the half-life of Env in KIF16B
98 knockout (KO) cells revealed an enhanced rate of Env degradation that could be reversed
99 using an inhibitor of lysosomal acidification. Knockout of KIF16B expression in a T cell
100 line diminished cell surface levels of Env incorporation, Env incorporation into particles,
101 particle infectivity, and viral replication in a spreading infection. These studies implicate
102 KIF16B in anterograde movement of Env away from degradation in the lysosome,
103 allowing more efficient incorporation into HIV-1 particles.

104

105 **RESULTS**

106 **Env localizes to KIF16B-positive compartments and is redistributed by**
107 **dominant-negative KIF16B overexpression.** To determine if HIV-1 Env is found in
108 KIF16B-positive endosomal compartments, we co-expressed the HIV-1 NL4-3 Env with
109 KIF16B tagged with yellow fluorescent protein (YFP) in HeLa cells. KIF16B+
110 compartments were readily observed at the periphery of cells and demonstrated a striking
111 colocalization with Env (Fig. 1A). Expression of KIF16B bearing a mutation in the
112 nucleotide binding motif that disrupts its motor function, KIF16B-S109A-YFP, has been

113 shown to act in a dominant-negative fashion to impair EGFR trafficking (14). Similar to
114 what was seen with EGFR, HIV-1 Env was redistributed by expression of KIF16B-S109A-
115 YFP to a perinuclear location (Fig. 1B). Expression of Env from intact NL4-3 proviral DNA
116 together with wildtype KIF16B led to a peripheral distribution of Env, similar to that seen
117 upon expression of Env alone (Fig. 1C). Expression of intact provirus with KIF16B-S109A-
118 YFP also revealed relocalization of Env to a perinuclear location (Fig. 1D). To quantify
119 the observed difference in subcellular localization, we used a mask 30% the distance from
120 the outer edge at the periphery of the cell to the stained nucleus, and measured Env
121 signal within this mask as compared to total Env signal in the cell. 40 cells were examined
122 for this analysis. The significant differences in peripheral localization shown in the
123 representative images were confirmed quantitatively, as shown in Fig. 1E. Expression of
124 either WT or S109A KIF16B resulted in a high degree of colocalization with Env, as
125 measured by Pearson's coefficient (Fig. 1F). We noted a somewhat higher degree of
126 colocalization with KIF16B-S109A-YFP expression, which was statistically significant
127 when Env alone was expressed (Fig. 1F). These results largely mirror results reported for
128 KIF16B relocalization of EGFR (14), and support the hypothesis that KIF16B promotes
129 anterograde transport of Env in early endosomal compartments.

130 **KIF16B fails to redistribute heterologous viral glycoproteins.** We considered the
131 possibility that overexpression of KIF16B could divert early endosomes to the periphery
132 of cells in a non-specific manner that would be seen with other viral envelope
133 glycoproteins. To address this possibility, we examined the effect of expression of WT vs.
134 S109A KIF16B on the distribution of three unrelated viral glycoproteins: influenza
135 glycoprotein hemagglutinin (HA), Ebola virus glycoprotein (EBOV GP), and severe acute

136 respiratory syndrome coronavirus 2 spike (SARS-CoV-2 S) protein. As noted previously,
137 WT KIF16B was consistently found at the periphery of cells, while KIF16B-S109A
138 localized near the cell nucleus. No redistribution of HA, EBOV GP, or SARS-CoV-2 S was
139 seen for KIF16B vs. KIF16B-S109A expression (Fig. 2A-F). Furthermore, in contrast to
140 HIV-1 Env, these heterologous viral glycoproteins failed to colocalize significantly with
141 KIF16B as indicated by visual inspection and by determination of a slightly negative
142 Pearson's Coefficient (supplemental Fig. S1C). These results demonstrate that HIV-1 Env
143 colocalization with and redistribution by KIF16B is not widely generalizable to viral
144 glycoproteins, at least as indicated by examination of a paramyxovirus, filovirus, and
145 coronavirus envelope glycoprotein. Next, we examined the effects of expression of
146 KIF1B, a closely related kinesin mediating plus end-directed trafficking, or its dominant
147 negative (motor-deficient) form KIF1B-Q98L (18), on HIV-1 Env distribution. KIF1B was
148 found at the cellular periphery, while and KIF1B-Q98L localized to perinuclear locations
149 in a manner similar to that of KIF16B and KIF16B-S109A (supplemental Fig. S1A, 1B).
150 However, distribution of HIV-1 Env did not appear to shift to the periphery or perinuclear
151 region upon expression of KIF1B and KIF1B-Q98L, respectively (Fig. S1A, 1B). In
152 contrast to results with KIF16B, Env was not significantly colocalized with either KIF1B
153 protein (Fig. S1C). Together, these results suggest that KIF16B plays a specific role in
154 anterograde trafficking of HIV-1 Env.

155 **HIV-1 Env on the PM is endocytosed into KIF16B positive endosomes.** The
156 studies described above examined the total pool of Env in the cell, and did not study the
157 endocytosed pool of Env in isolation. In order to determine if Env is delivered into KIF16B
158 positive endosomes directly from the PM, we utilized a novel approach using flourogen

159 activating protein (FAP) tagging (19). The FAP tag was placed within the V2 loop of the
160 ectodomain of JR-FL Env as illustrated in Fig. 3A, creating the FAP-Env construct. To
161 validate this system for use in assessing Env incorporation, we examined particle
162 incorporation of FAP-Env. FAP-Env was co-expressed with Env-deficient provirus, and
163 was efficiently incorporated into virions as indicated by Western blot analysis
164 (supplemental Fig. S2). Combining expression of this construct (FAP-Env) with a
165 flourogen impermeant to the PM (MG-11p, αRED-np1 from Spectragenetics) allowed
166 visualization of the fate of pulse-labeled Env present on the surface of cells. We first co-
167 expressed FAP-Env with KIF16B WT in HeLa cells and fixed the cells after pulse labeling
168 them on ice to visualize the surface staining in the absence of endocytosis. Fig. 3B
169 demonstrates that the cell membrane-impermeable reagent fluoresced upon binding to
170 Env on the cell surface and did not penetrate the PM. We next allowed internalization of
171 FAP-Env for 15 minutes at 37°C after pulse-labeling, and found that surface-labelled FAP-
172 Env on the surface had accumulated into KIF16B-positive compartments (Fig. 3B). We
173 also performed the same experiment with KIF16B S109A and found similar results (Fig.
174 3C). In order to follow the movement of this FAP-Env population expressed on the cell
175 surface in real-time, we pulse-labeled FAP-Env and recorded live-cell video of Env
176 movement into HeLa cells expressing KIF16B or KIF16B-S109A. Pulse-labeled Env was
177 endocytosed within a few minutes and demonstrated increasing colocalization with
178 KIF16B+ endosomes at the periphery of cells, or with KIF16B-S109A+ endosomes in the
179 perinuclear region of the cell (Fig. 3D and 3E, and supplemental video 1 and 2). These
180 results demonstrate that HIV-1 Env on the surface of the cell is rapidly endocytosed and
181 thereafter accumulates in KIF16B+ endosomes.

182 **Deletion of KIF16B leads to enhanced degradation of Env and accumulation**
183 **within the lysosome.** It has been established that KIF16B-mediated transport can divert
184 cellular cargo from default pathways that lead to lysosomal degradation, while depletion
185 of KIF16B enhances their degradation (14, 16) We therefore hypothesized that a loss of
186 normal anterograde trafficking of Env mediated by KIF16B could result in enhanced Env
187 degradation. To evaluate this possibility, we utilized CRISPR/Cas9 technology to
188 knockout KIF16B in both HeLa cells and H9 T cells, followed by characterization of
189 isolated cell clones. Fig. S3 shows characterization of one HeLa and one H9 clone used
190 for subsequent studies, and is consistent with the findings from several clones examined
191 for each cell type. Note the complete absence of KIF16B by Western blotting in
192 supplemental Fig. S3A. We then utilized the KIF16B KO HeLa cell clones together with
193 pulse-labeling of Env using the FAP tag technique described above. FAP-Env was
194 expressed in WT or KIF16B KO cells, and the kinetics of disappearance of total cellular
195 FAP-Env signal was measured over time. These experiments were controlled for the
196 effects of photobleaching on FAP-Env signal as outlined in Materials and Methods.
197 Remarkably, the FAP-Env signal degraded at a considerably faster rate in KIF16B KO
198 cells than in WT cells (images in Fig. 4A vs. Fig. 4B and supplemental video 3). The half-
199 life of endocytosed Env measured in WT HeLa cells by this method was 159.5 minutes
200 (95% C.I. 145.4 – 175.6), while in the absence of KIF16B Env was more rapidly degraded,
201 with a half-life of 56.9 minutes (95% C.I. 54.1 – 59.8). Furthermore, when we incubated
202 KIF16B KO cells with lysosomal inhibitor bafilomyacin-A1 (Baf-A1), the degradation rate
203 in KIF16B KO cells returned to wild-type levels (Fig. 4 A, B, C, E and supplemental video
204 3). To corroborate the observation that KIF16B knockout leads to enhanced degradation

205 of Env as measured by degradation of FAP-tagged Env, we performed surface pulse-
206 labeling with NHS-biotin in WT and KIF16B KO HeLa cells on ice, followed by quenching
207 of remaining biotin and pulldown of internalized biotinylated proteins with streptavidin
208 beads at multiple timepoints following incubation at 37°C. Detection of internalized Env
209 was then performed using quantitative Western blotting. Figure 4D shows Western blot
210 examples representative of results from five individual experiments for each experimental
211 arm. As compared with WT cells, pulse-labeled Env in KIF16B KO cells was more rapidly
212 degraded (Fig. 4D, compare WT to KO blots). The addition of Baf-A1 reduced the
213 degradation to essentially WT levels (Fig. 4D, 4F). Using repeated experiments, we
214 generated degradation curves for pulsed Env in WT, KO, and KO + Baf-A1 cells that were
215 similar, but not identical, to the curves derived from FAP-Env labeling (Fig. 4F, compare
216 to 4E). Env half-life measured by this method was 154.1 minutes for WT cells, 32.1
217 minutes for KO cells, and 94.9 minutes for Baf-A1-treated KO cells. While we noted that
218 the results were not identical between the two methods employed, they are in good
219 general agreement, and establish that KIF16B KO leads to more rapid Env degradation
220 that can be rescued by Baf-A1. Together, these results clearly show that KIF16B limits
221 lysosomal degradation of Env, likely through promoting anterograde trafficking.

222 Given results above with the lysosomal inhibitor Baf-A1, we sought to determine if the
223 absence of KIF16B results in a shift in Env distribution into the lysosomal compartment.
224 Results of immunofluorescence staining were striking. WT HeLa cells revealed punctate
225 lysosomes with a moderate degree of Env colocalization, while intracellular Env was
226 clearly distinguished outside of the lysosome (marked by LAMP1 staining) (Fig. 5A). In
227 KIF16B KO cells, Env colocalization with LAMP1 was dramatically higher (Fig. 5B and

228 5D). Baf-A1 treatment of KIF16B KO cells did not show significant differences in
229 colocalization of Env and LAMP1 as compared with untreated KO cells, as both showed
230 very high levels of colocalization as quantified by Pearson's coefficient (Fig. 5C and 5D).
231 Taken together, these results from imaging and pulse-chase analysis suggest that
232 deletion of KIF16B increases the rate of Env degradation due to loss of anterograde
233 movement normally conferred by KIF16B, resulting in shunting to the lysosome for
234 degradation.

235 **KIF16B deletion leads to defects in Env incorporation, particle infectivity, and**
236 **viral replication.** We next examined the effect of KIF16B depletion on Env trafficking and
237 incorporation into particles. We first assessed surface levels of Env by flow cytometry
238 after infecting WT or KIF16B KO H9 cells with NL4-3 virus. Surface expression levels of
239 Env were consistently diminished in KIF16B KO cells compared with WT cells (Fig. 6A
240 and 6B), consistent with diminished anterograde movement of Env. Total cellular Env
241 measured by flow cytometry was also diminished, but to a somewhat lesser degree than
242 cell surface Env (Fig. 6B). This result is consistent with the enhanced lysosomal
243 degradation of endocytosed Env shown in prior results. Viruses were then purified from
244 supernatants of infected WT and KIF16B KO H9 cells, in order to examine levels of Env
245 incorporation into particles. Viral particle Env content was significantly reduced in KIF16B
246 KO cells compared to WT H9 (>50% reduction from wild-type levels as quantified by LI-
247 COR Image Studio Software, n = 5) (Fig. 6C). The leftmost blot in Fig. 6C shows particle
248 Env content normalized by equal p24 content loading of the lanes. When cell lysates were
249 examined following multiple rounds of replication in WT or KIF16B KO cells and
250 normalized for total protein, there was a reduction of all viral proteins, reflecting

251 diminished replication (Fig. 6C, cell lysate). Notably, however, production of gp160 in
252 KIF16B KO cells was not diminished, as indicated by Western blot of lysates from infected
253 cells examined after a single round of replication (Fig. 6C, rightmost blot). This suggests
254 that the reduced levels of Env seen on the PM and on particles derived from KIF16B KO
255 cells were not due to an Env production defect. To evaluate the significance of the
256 diminished particle incorporation of Env induced by KIF16B KO, we next examined
257 particle infectivity and viral replication. Consistent with the observed reduction of Env on
258 particles, the infectivity of particles released from KIF16B KO cells was consistently
259 reduced (Fig. 6D). We noted that the reduction in Env incorporation shown in Fig. 6C and
260 the reduction in particle infectivity in 6D were consistent but are incomplete, suggesting
261 that although KIF16B alters the level of Env incorporation, it is not absolutely required for
262 incorporation of Env into particles. To determine the significance of this level of Env
263 reduction on viral replication, we infected WT H9 and KIF16B KO cells and measured
264 viral replication in a spreading infection. Infection of KIF16B KO H9 cells resulted in a
265 significant delay in replication and a reduced peak of released virus as compared with
266 WT H9 cells (Fig. 6E). We conclude from these results that the anterograde movement
267 of Env by KIF16B plays a role in Env incorporation and viral replication. Loss of KIF16B
268 results in lower levels of cell surface Env and a corresponding loss of Env incorporation
269 into particles.

270

271 **DISCUSSION**

272 Molecular motor proteins are essential to the normal function of eukaryotic cells. The
273 cytoplasm of cells is dense, crowded with proteins, vesicles, nucleotides, and organelles.

274 Free diffusion of large molecules within the cytoplasm is restricted, and cannot be relied
275 upon to move these cellular materials with the efficiency and accuracy required for proper
276 cell function. Instead, the cell must use specialized molecular motors. These motors –
277 kinesins, dyneins, and myosins – use adenosine triphosphate (ATP) to power movement
278 of cargo along cytoskeletal structures. Kinesins and dyneins utilize microtubule networks
279 as their pathways for transporting cellular cargo. With some exceptions, kinesins move in
280 an anterograde direction along microtubules. Dyneins, in contrast, move in a retrograde
281 direction toward the minus end of microtubules often localized at the microtubule-
282 organizing center (MTOC). Kinesins and dyneins are most commonly structured with an
283 N-terminal motor head that is responsible for powering processive, step-like movement
284 of motor subunits, attached via a middle stalk region to a C-terminal tail composed of light
285 and heavy chains that bind the cellular cargo (20-23) .

286 Viruses hijack host cellular pathways in order to perform essential steps in their
287 lifecycle, including co-opting molecular motor-driven intracellular transport (24-27). HIV-1
288 has been shown to utilize motor proteins at several steps of replication (27, 28). Following
289 entry to a cell, the conical core of HIV-1 has been shown to bind to dynein and to use the
290 KIF5 motor protein to successfully traffic towards the nucleus (29, 30). Depletion of the
291 KIF5B motor prevents nuclear entry and infection of HIV-1 in a CA-dependent manner
292 (31). In studies of outward trafficking of Gag, KIF4 has been shown to directly interact
293 with Gag in both yeast two-hybrid and co-immunoprecipitation assays (32, 33). Disruption
294 of KIF4 slowed progression of Gag through trafficking intermediates, and depletion of
295 KIF4 increased degradation of Gag. The increased degradation of Gag was reversed
296 upon reintroduction of KIF4 (34). However, no motor protein has previously been shown

297 to interact with the HIV-1 Env protein or participate in its trafficking within host cells.
298 Because Env that reaches the cell surface through the secretory pathway is rapidly
299 endocytosed and must subsequently traffic in an anterograde manner to return to the PM
300 for particle incorporation, we hypothesized in this study that HIV-1 would also likely use
301 a kinesin or kinesins for outward trafficking of Env. Several pieces of evidence implicate
302 Rab14 as an important outward trafficking factor for Env. Our group has shown that
303 Rab14 mediates FIP1C-dependent recycling of Env and plays a role in Env incorporation
304 (10). Hoffman and colleagues similarly found that Env colocalizes to Rab14+ endosomal
305 compartments in T cell lines, and unexpectedly found that these compartments shared
306 some characteristics with late endosomes and lysosomes (13). KIF16B is an effector of
307 Rab14-mediated trafficking that binds directly to Rab14 (15). In this study, we
308 demonstrate that KIF16B plays a role in Env trafficking and in the ability of HIV-1 to form
309 fully infectious particles. Similar to other reported cargo of KIF16B such as EGFR, Env
310 colocalized strongly with KIF16B at the periphery of cells. Motor mutant dominant
311 negative KIF16B redistributed Env to a perinuclear position, entirely consistent with what
312 had been seen for EGFR. Video microscopy of pulse-labeled Env expressed on the
313 surface of cells in this study indicated that Env on the PM is endocytosed into KIF16B-
314 positive compartments. In the absence of KIF16B, Env was shifted to the lysosome, and
315 the half-life of Env that had been endocytosed from the PM was significantly reduced.
316 Taken together, these studies strongly suggest that KIF16B mediates anterograde
317 movement of Env and limits the pool of Env that is shunted to the lysosome for
318 degradation. To our knowledge, this is the first evidence for a motor protein contributing
319 to Env trafficking and particle incorporation.

320 Data from this study support the model shown in Fig. 7. According to this model, Env
321 is transported through the secretory pathway to the PM and subsequently endocytosed
322 to the early or recycling compartments, following which Env can be directed in an
323 anterograde fashion by KIF16B or can follow a default pathway leading to fusion with the
324 lysosome (Fig. 7A). In the absence of KIF16B, a larger pool of endocytosed Env is
325 shunted to the lysosome, as indicated by the relative size of the arrows in Fig. 7B. The
326 fraction of the endocytosed pool of Env that is directed to the periphery and is
327 subsequently available for incorporation into particles is diminished in the absence of
328 KIF16B, resulting in diminished particle incorporation, particle infectivity, and reduced
329 replication. Fig. 7 for simplicity does not include other potential trafficking routes of Env,
330 such as retrograde trafficking to the Golgi and the potential for alternative routes for Env
331 to recycle to the PM.

332 Four models for Env incorporation into HIV particles have been proposed by the Freed
333 laboratory (5). Passive incorporation at the PM is not likely to be the correct model in
334 nonpermissive cell types including primary T cells and macrophages, as Env with a full-
335 length CT is excluded in these cell types. This model remains a potential in permissive
336 cell lines that do not require the CT (4). The identification of host factors such as Rab11-
337 FIP1C, Rab14, and KIF16B that influence particle incorporation favors the Gag-Env co-
338 targeting model, whereby Env is directed to specific microdomains on the PM that are
339 enriched in Gag molecules and serve as sites of particle budding. At present there is no
340 evidence favoring an indirect interaction model with an adaptor or linker connecting Gag
341 to Env. The direct Gag-Env interaction model has been supported by two biochemical
342 studies (35, 36), and by evidence that maturation is required before Env can mediate

343 membrane fusion, while CT truncation relieves the constraint on fusion posed by the
344 immature Gag lattice (9, 37, 38). Trimerization of MA has been shown to be required for
345 Env incorporation, while some mutations in MA that result in Env exclusion from particles
346 can be rescued by compensatory mutations at the trimer interface (39, 40). Furthermore,
347 MA trimerization-impaired mutants can be rescued by substitutions that simultaneously
348 restore Env incorporation (41). This has added substantially to the evidence favoring an
349 interaction of the CT with MA in the form of MA trimers, and supports a model whereby
350 the structural integrity of the MA lattice is an important determinant of Env incorporation
351 (41). Results in the present study showing a role for kinesin-mediated trafficking of Env
352 do not negate the importance of structural integrity of the MA lattice for particle
353 incorporation of Env. Trafficking to the site of the developing Gag lattice mediated by host
354 factor interactions with the CT may be a necessary step preceding a direct Gag-Env
355 interaction. We expect that a defect in anterograde trafficking and increase in Env
356 degradation in the lysosome would diminish the delivery of Env to sites of assembly and
357 reduce the potential interaction of Env with the Gag lattice.

358 This study provides new information regarding the half-life of Env trimers following
359 endocytosis from the PM. A prior study employing autoradiographic pulse-labeling of total
360 cellular Env indicated that cellular gp160 and gp120 are degraded over a period of hours,
361 and that inhibition of lysosomal acidification prevents gp160 degradation (42).
362 Degradation of gp160 has been shown to not only occur in the lysosome, but also within
363 the biosynthetic pathway within the ER or Golgi compartments (43, 44). However, these
364 studies labeled all Env populations within the cell, whereas the current study utilized two
365 methods of cell surface pulse-labeling followed by chase periods to measure the half-life

366 of Env trimers that had reached the plasma membrane. We found that endocytosed Env
367 has a half-life of 150-160 minutes, and that the absence of KIF16B promotes much more
368 rapid degradation (57 or 32 minutes, measured by FAP-Env degradation or surface
369 biotinylation, respectively). An important role for lysosomal degradation of endocytosed
370 Env is suggested by the reversal of the enhanced rates of degradation in KIF16B KO cells
371 by Baf-A1. A model in which endocytosed Env is shunted to the lysosome for degradation
372 in the absence of KIF16B is also supported by the increased appearance of Env in
373 lysosomal compartments in KIF16B KO cells.

374 An important caveat for our conclusions is that a complete KIF16B KO resulted in an
375 incomplete reduction of Env incorporation and particle infectivity. Env incorporation and
376 particle infectivity were diminished by approximately 50%. This suggests that kinesins
377 other than KIF16B may also be involved in trafficking Env to the PM, or that there are
378 multiple pathways whereby Env may be recycled to the surface. Indeed, it is known that
379 successful movement of cargo within cells is often accomplished by groups of motors
380 rather than just one (45). Retrograde trafficking of Env from early endosomes to the Golgi
381 may play a role in Env incorporation into particles, as depletion of components of the
382 retromer complex modulated Env distribution and particle incorporation (46). However,
383 the identification of KIF16B as a regulator of Env trafficking and degradation defines
384 another component of the host machinery regulating Env incorporation. Kinesins have
385 been proposed as potential therapeutic targets in some areas of medicine such as cancer
386 and chronic pain (47-49). Although targeting KIF16B function globally would be disruptive
387 to many cell processes, it is possible that a more directed approach disrupting KIF16B-
388 mediated Env trafficking can be developed. In conclusion, we identify KIF16B as an

389 anterograde motor involved in Env trafficking and particle incorporation. Future studies
390 will be needed to fully elucidate the complex pathways taken by Env to reach the site of
391 particle assembly and enable particle incorporation.
392

393 **MATERIALS AND METHODS**

394 **Cells and Plasmids.** HeLa cells were obtained from the American Type Culture
395 Collection (ATCC; CRM-CCL-2). TZM-bl cells were obtained through the NIH AIDS
396 Reagent Program, Division of AIDS, NIAID, NIH, from John C. Kappes, Xiaoyun Wu, and
397 Tranzyme, Inc. The H9 T cell line was obtained from ATCC (HTB-176), and cells were
398 maintained in RPMI medium 1640 supplemented with 10% fetal bovine serum (FBS), 2
399 mM Glutamine, and antibiotics. HeLa and TZM-bl cells were maintained in DMEM
400 (Dulbecco's modified Eagle medium) containing 10% FBS and 2 mM penicillin-
401 streptomycin. pNL4-3 proviral plasmid was obtained through the NIH AIDS Reagent
402 Program, Division of AIDS, NIAID, NIH, from Malcolm Martin. Env expression plasmid
403 pIIINL4Env was a gift kindly provided by Dr. Eric Freed (HIV DRP, NCI, NIH) (6). The
404 Influenza A vaccine strain of A/Brisbane/02/2018 (H1N1) HA sequence was used to
405 construct a human codon-optimized gene which was synthesized and cloned into the
406 BamHI and EcoRI sites of pcDNA3.1+ by GenScript. The SARS-CoV-2 spike gene
407 (Wuhan) was synthesized by Genscript for Dr. Biao He (University of Georgia) and gifted
408 to our lab. The human codon-optimized full length SARS-CoV-2 spike gene was
409 subcloned into the KpnI and BamHI sites of the plasmid pcDNA5TO/Puro. pcDNA5/TO-
410 puro was constructed from backbone pCDNA5/TO (ThermoFisher Scientific) as
411 previously described (50). The EBOV S protein plasmid pcDNA5/TO-puro EBOV GP has

412 been previously described (51). Plasmid pcDNA5/TO JR-FLoptgp160 expresses a
413 codon-optimized JR-FL *env* gene in the pcDNA5/TO vector backbone (Thermo Fisher
414 Scientific, Waltham, MA) as previously described (12). Overlap-extension PCR was used
415 to create novel Xho1 and Pst1 sites within the exposed portion of the V2 loop of JR-FL.
416 The fluorogen-activated peptide (FAP) sequence from pMFAP-alpha2 (Spectragenetics,
417 Pittsburgh, PA) was PCR amplified as an Xho1-Pst1 fragment and ligated into
418 pcDNA5/TO JR-FLoptgp160, placing the FAP peptide in-frame within the V2 loop. A
419 complete cloning schema with primers is available upon request. The WT KIF16B-YFP
420 and dominant KIF16B-S109A-YFP were kindly provided by Sebastian Hoepfner (14).
421 Expression plasmids for Flag-KIF1Bβ and Flag- KIF1Bβ Q98L were kindly provided
422 Susanne Schlisio and described in Fell et. al. 2017 (18).

423 **Viral infectivity and multi-round replication assays.** H9 T cells were infected with
424 VSV G-pseudotyped NL4-3 virus at an MOI of 0.2. Cells and supernatants were harvested
425 4-5 days after infection for analysis by Western blotting, p24 antigen quantitation, and
426 infectivity assays. Infectivity of cell culture supernatants was measured using TZM-bl
427 indicator cells following p24 normalization as previously described (52). Replications
428 assays were performed by taking aliquots of the supernatant after infection every 3 days
429 for 21 days. Supernatant was then used in a p24 ELISA assay to graph the amount of
430 virus produced over the course of viral growth and replication. Murine anti-p24 capture
431 antibody 183-H12-5C (CA183) was obtained from Bruce Chesebro and Kathy Wehrly
432 through the NIH AIDS Research and Reference Reagent Program, and the p24 ELISA
433 assay was performed as previously described (53).

434 **Western blotting, antibodies, and immunostaining.** For analysis of viral protein
435 content, viruses were pelleted from cell culture supernatants through a 20% sucrose
436 cushion at 35,000 × g for 3 h at 4°C; cells were harvested and pelleted at 400 × g. Virus
437 pellets and cell lysates were dissolved in radioimmunoprecipitation assay (RIPA) buffer
438 (Thermo Scientific) containing protease inhibitors. Cell lysates for blotting KIF16B were
439 prepared with a hypotonic lysis buffer consisting of 10mM HEPES, 1.5mM $MgCL_2$, 10 mM
440 KCl in PBS on ice for 15 minutes, vortexed with 10% IGEPAL CA630 (Sigma) to break
441 open cells, and then centrifuged to pellet out nuclei. Virus pellets and cell lysates were
442 separated on 4 to 12% polyacrylamide gels, transferred to nitrocellulose, and subjected
443 to Western blotting using antibodies outlined below. Goat polyclonal antibody AHP2204,
444 from Bio-Rad AbD Serotec (Oxford, UK)(D7324), was used for detecting HIV-1 gp120 for
445 Western blotting. Recombinant human monoclonal antibody 2F5 from Polymun scientific
446 (Klosterneuburg, Austria) was used to detect HIV-1 gp41 in Western blots. Actin was
447 detected with Invitrogen Actin antibody ACTN05. KIF16B antibody used was polycolonal
448 mouse from Abnova (H00055614-B01P). Mouse anti-p24 monoclonal CA-183 (provided
449 by Bruce Chesebro and Kathy Wehrly through the NIH AIDS Research and Reference
450 Reagent Program) was used to detect p24 and $Pr55^{Gag}$ in Western blotting and for
451 enzyme-linked immunosorbent assay (ELISA) to measure HIV-1 Gag in virus-containing
452 culture supernatant and virus pellets; the capture ELISA was performed as previously
453 described (54). IRDye goat anti-mouse, IRDye rabbit anti-goat, and IRDye goat anti-rabbit
454 secondary antibodies from LiCor Biosciences (Lincoln, NE) were used for Western
455 blotting. All blots were acquired and analyzed using the LiCor Odyssey infrared detection
456 system. The following antibodies were utilized in immunofluorescence assays: human

457 anti-gp120 antibody IgG1 2G12 from Polymun scientific (A002), monoclonal ANTI-
458 FLAG® antibody produced in rabbit from Sigma-Aldrich (F2555), rabbit polyclonal anti-
459 HA1 antibody from Sino Biological (Beijing, China) (11692-T54), mouse monoclonal
460 SARS-CoV / SARS-CoV-2 (COVID-19) spike antibody from Genetex (Irvine, CA) (1A9),
461 mouse anti- EBOV GP monoclonal antibody 4F3 from IBT Bioservices (Rockville, MD),
462 anti-LAMP1 mouse monoclonal antibody from BD Biosciences (Franklin Lakes, NJ)
463 (H4A3).

464 **Env degradation assays.** In order to determine degradation rates of Env
465 microscopically using our FAP-tagged Env, we plated 100k HeLa WT or KIF16B KO HeLa
466 cells in wells of Ibidi 4 well μ-Slide plate (Ibidi) and then transfected overnight with the
467 FAP-Env construct. In one of the KO cell wells, 100μM concentration of Bafilomycin A1
468 (Baf-A1, Invivogen) was employed while the WT and other KO cell wells received an
469 equal amount of DMSO. Three hours later, live cell imaging was conducted following a
470 10 min pulse label incubation with FAP reagent αRED-np1 (Spectragenetics) in 5% CO_2
471 at 37°C chamber on Widefield Nikon Ti2 inverted SpectraX (Cincinnati Children's
472 Confocal Core). Data from these movies was extracted on Volocity imaging software
473 (Quorum Technologies) to acquire the signal intensity of each ROI drawn around
474 individual cells analyzed in the field of view over the time course. The averaged
475 normalized signal intensity was then calculated using the method outlined in Alber et. al.
476 to attain the degradation curve and half-life of the FAP-tagged Env protein (55). For
477 protein analysis of Env degradation by Western blotting, we plated 1.75 million WT or
478 KIF16B KO HeLa cells in 10cm dishes (5 for WT, 10 for KO). The next morning, 3ug of
479 pIII-Env and 250 ng of psv72-Tat protein were cotransfected into each plate. Following

480 24 hours of incubation, 100μM Baf-A1 was added to 5 of the KO dishes, while the other
481 KO and WT dishes received an equal amount of DMSO. Three hours later a 1 mM solution
482 of biotin/PBS was applied to the cells and allowed to label surface proteins for 15 min.
483 This labeling reaction was quenched with 1x TBS solution for 5 min. Cells were then
484 washed with PBS and returned to normal media. Cell plates were then put on ice and in
485 a 4°C cold room to stop endocytosis at 0, 30, 60, 90, 120 min after biotin labeling and
486 were then subsequently scrapped and harvested for cell lysates in RIPA buffer. Cell
487 lysates were then IP overnight with 60μL of Pierce™ High Capacity NeutrAvidin™
488 Agarose (Thermo-Fischer). Beads were then collected in Pierce™ Spin Columns
489 (Thermo-Fischer) and protein was then eluted off the beads at 95°C in 1x Laemmli buffer
490 with reducing agent. After 2000 x g spin for 3 min equal amounts of elution were run for
491 each time point on a 4-12% SDS-Page gel and then analyzed by immunoblotting for
492 gp120.

493 **Image acquisition and analysis.** HeLa cells were plated in MatTek 35mm poly–d-
494 lysine-treated dishes (Brooke Knapp MatTek) overnight and then were then transfected
495 with the indicated constructs using jetPRIME (Polyplus) per manufacturer's instructions.
496 After a 24 h incubation, cells were rinsed with phosphate-buffered saline (PBS) three
497 times and fixed in 4% paraformaldehyde for 10 min at room temperature. Cells were
498 washed with PBS three times with mild agitation after fixation, and then permeabilized for
499 10 min with 0.2% Triton X-100, followed by washing with PBS. Dako blocking solution
500 was applied to cells for 30 min under gentle shaking conditions. Cells were then incubated
501 with the appropriate primary antibody detailed above diluted in Dako (Agilent) antibody
502 diluent to 1:500. Species appropriate Alexa-flour (Thermo-Fischer) secondary antibody

503 was diluted in Dako antibody diluent to 1:1,000. Images were acquired on a widefield
504 DeltaVision Elite High Resolution microscope (GE Healthcare). Percent of total Env signal
505 at the periphery of cells was calculated using measurements from Volocity 6.5.1 imaging
506 analysis software. Briefly, regions of interest were drawn around the entire cell, around
507 30% of the distance from the edge of the cell signal to the nucleus (inner 70% of total cell
508 area), and around the nucleus. Peripheral signal was calculated by subtracting the middle
509 two regions from the sum of signal from the total cell region, giving a percentage of
510 peripheral signal as all signal in the last 30% of the cell body towards the edge.
511 Quantitation of colocalization was performed using Volocity software features by
512 derivation of Pearson's correlation coefficient after manual thresholding.

513 **Flow cytometry for HIV-1 Env expression on cell surface.** For cell surface staining,
514 approximately 5 million H9 cells were infected overnight with 1 μg p24 of VSV-G-
515 pseudotyped NL4.3. The next day, cells were washed once with PBS and plated in a six
516 well plate in RPMI for 48 hours. Infected cells were stained for viability with zombie violet
517 dye (BioLegend) diluted 1:500 in PBS, followed by fixation with 4% paraformaldehyde.
518 Cells were blocked with Dako protein block then stained for cell surface Env with 2G12
519 anti-gp120 directly conjugated to APC (Abcam, ab201807) and diluted 1:100 in Dako
520 antibody diluent (10ug/mL final concentration) for 2 hours. 2G12 was washed out twice
521 with PBS followed by permeabilization with 0.2% triton-X 100. Lastly, cells were stained
522 for total infected cells using p24 antibody, KC-57 FITC diluted 1:100 in Dako antibody
523 diluent. Cells were washed twice with PBS then resuspended in MACS (Miltenyi Biotec)
524 buffer and analyzed using the BD FACS Canto II.

525 **CRISPR KO strategy.** CRISPR-Cas9 technology was used to knockout KIF16B in
526 both HeLa and H9 cell lines. Using the Crispor.tefor.net open access website and
527 algorithm designed to find areas of the genome to target with guide RNAs (gRNA), we
528 targeted Exon 13 of KIF16B using sequence from NCBI (Gene ID 55614, Assebmly
529 GCF_000001405.40, Chr 20, location NC_000020.11) and found the stretch of DNA with
530 the highest score to target for deletion being CTCACGGATAAGTTTGACGT. We ordered
531 sense (CACCGCTCACGGATAAGTTTGACGT) and anti-sense
532 (AAACACGTCAAACTTATCCGTGAGC) gRNA oligos + PAM sequence from Integrated
533 DNA Technologies (IDT) to clone into the lentiCRISPR v2 that was a gift from Feng Zhang
534 (Addgene plasmid # 52961 ; http://n2t.net/addgene:52961 ; RRID:Addgene_52961) using
535 the target guide sequence protocol from Sanjana Lab
536 (http://sanjanalab.org/library/protocol_lentiOligo.pdf) (56) After transformation of MAX
537 Efficiency™ Stbl2™ Competent Cells (Thermo-Fischer) and DNA harvest, we packaged
538 the plasmid into a lentivirus using sPAX2 and PMGD2 and transduced our HeLa and H9
539 cell lines. Puromycin selection and limiting dilution were used to obtain clones knocked
540 out for KIF16B (Fig. S1).
541

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548 Imaging Center at CCHMC. DNA sequencing was performed at the DNA Sequencing
549 core at CCHMC.

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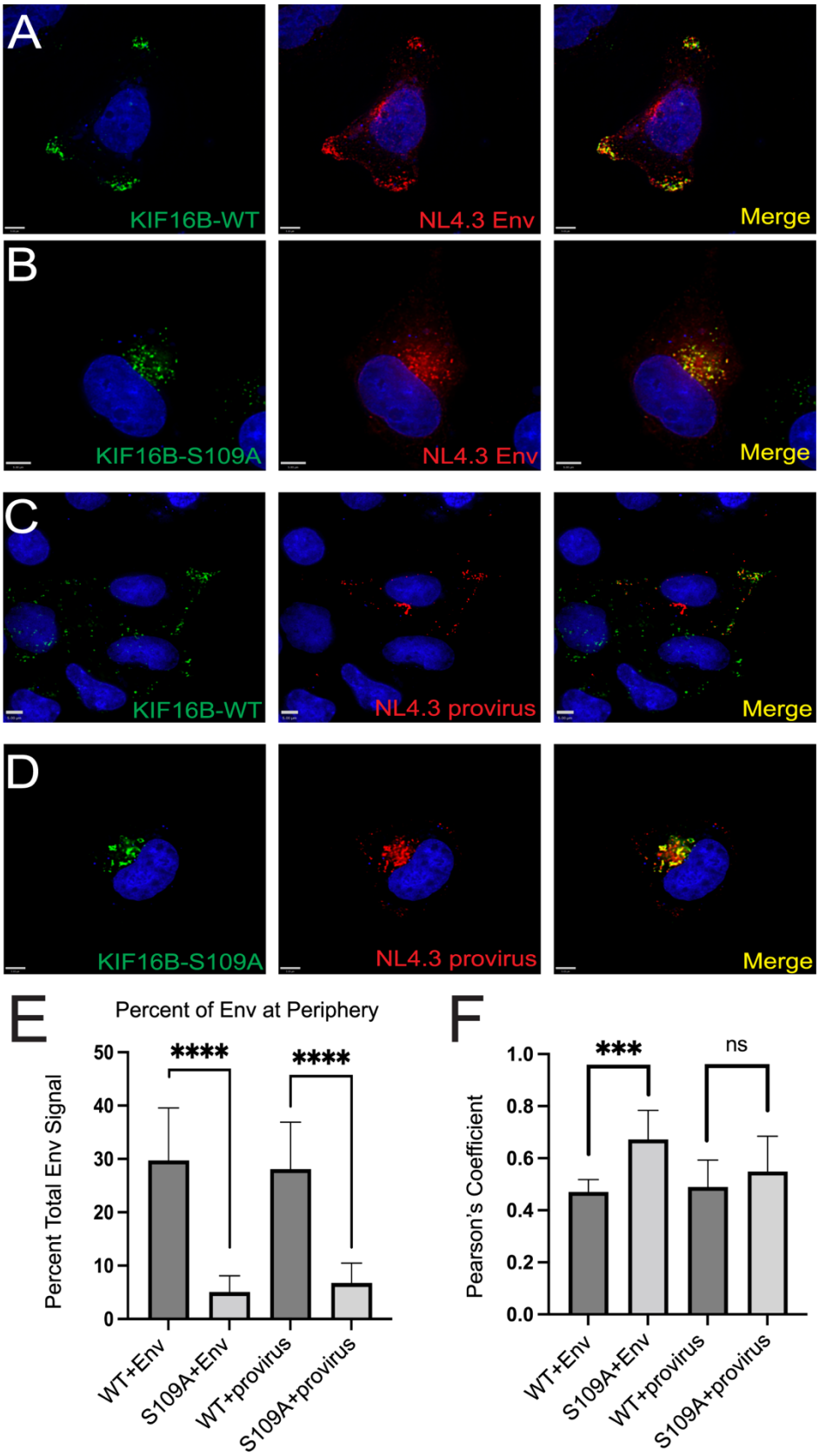
A
KIF16B-WT
NL4.3 Env
Merge
B
KIF16B-S109A
NL4.3 Env
Merge
C
KIF16B-WT
NL4.3 provirus
Merge
D
KIF16B-S109A
NL4.3 provirus
Merge
E
Percent of Env at Periphery
Percent Total Env Signal
0
10
20
30
40
50
****
****
WT+Env
S109A+Env
WT+provirus
S109A+provirus
F
Pearson's Coefficient
0.0
0.2
0.4
0.6
0.8
1.0
***
ns
WT+Env
S109A+Env
WT+provirus
S109A+provirus

717

718 **FIG 1** Env localizes to KIF16B-positive compartments and is redistributed by dominant-
719 negative KIF16B. (A) Subcellular distribution of NL4-3 Env when coexpressed with
720 KIF16B-WT-YFP. Cells were fixed and immunolabeled with human neutralizing antibody
721 2G12 to stain HIV-1 gp120. Green, KIF16B; red, Env; rightmost image, overlay. (B)
722 Distribution of NL4-3 Env when coexpressed with dominant KIF16B-S109A-YFP. Staining
723 is as described for panel A. (C and D) Coexpression of proviral NL4-3 with WT and
724 dominant-negative KIF16B, respectively. Staining for Env as described from A, B. (E)
725 Percent of Env signal at periphery of cells observed for each combination of coexpression
726 shown in A-D. Signal at the periphery was the sum all Env signal within 30% of the
727 distance from edge of cell to the nucleus. Results are shown as means ± SD from a total
728 of 10 representative images. ns = not significant; ***, $P < 0.0008$; ****, $P < 0.0001$. P values
729 were calculated using the Student's unpaired t test using GraphPad Prism 9. (F) The
730 degree of colocalization between KIF16B constructs and Env was measured by
731 Pearson's correlation coefficient equation using Volocity 6.5.1 software after thresholding.
732 Bars represent 5 μm.

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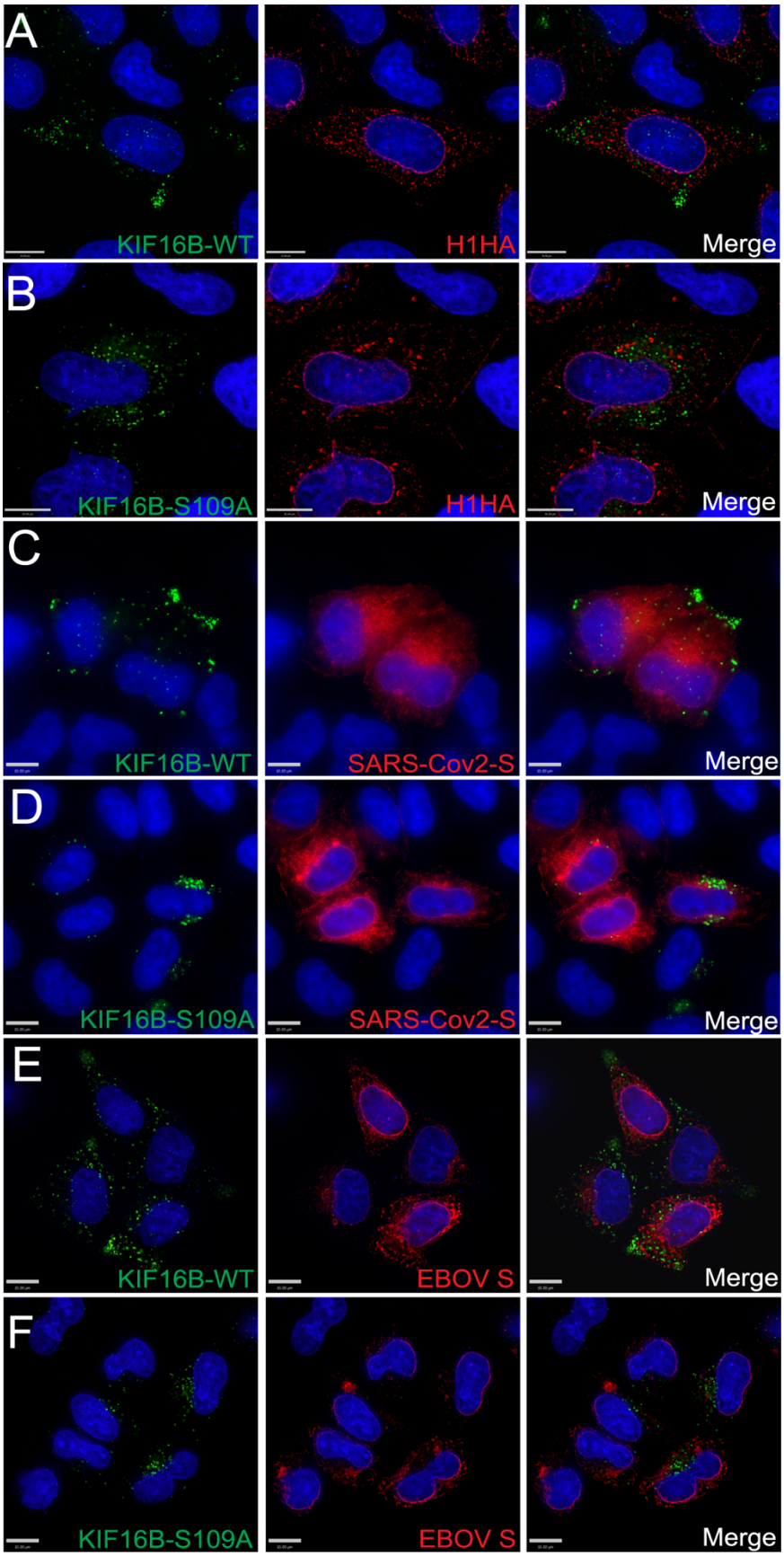
A
KIF16B-WT
H1HA
Merge
B
KIF16B-S109A
H1HA
Merge
C
KIF16B-WT
SARS-Cov2-S
Merge
D
KIF16B-S109A
SARS-Cov2-S
Merge
E
KIF16B-WT
EBOV S
Merge
F
KIF16B-S109A
EBOV S
Merge

739

740 **FIG 2** KIF16B and Env colocalization is not generalized to other viral envelope
741 glycoproteins. (A, B) Influenza HA glycoprotein was coexpressed with KIF16B-WT-YFP
742 and KIF16B-S109A-YFP, respectively. Cells were fixed and immunolabeled with 11692-
743 T54 antibody to stain HA. Green, KIF16B; red, HA; rightmost image, overlay. (C, D)
744 SARS-Cov2-S protein was coexpressed with WT and dominant negative KIF16B,
745 respectively. Cells were fixed and immunolabeled with 1A9 to stain spike. Green, KIF16B;
746 red, SARS-Cov2-S; rightmost image, overlay. (E, F) Coexpression of KIF16B-WT-YFP or
747 KIF16B-S109A-YFP with Ebola GP. Cells were fixed and immunostained with 4F3
748 antibody specific to EBOV GP. Green, KIF16B; red, EBOV GP; rightmost image, overlay.
749 Bars represent 10 μm.

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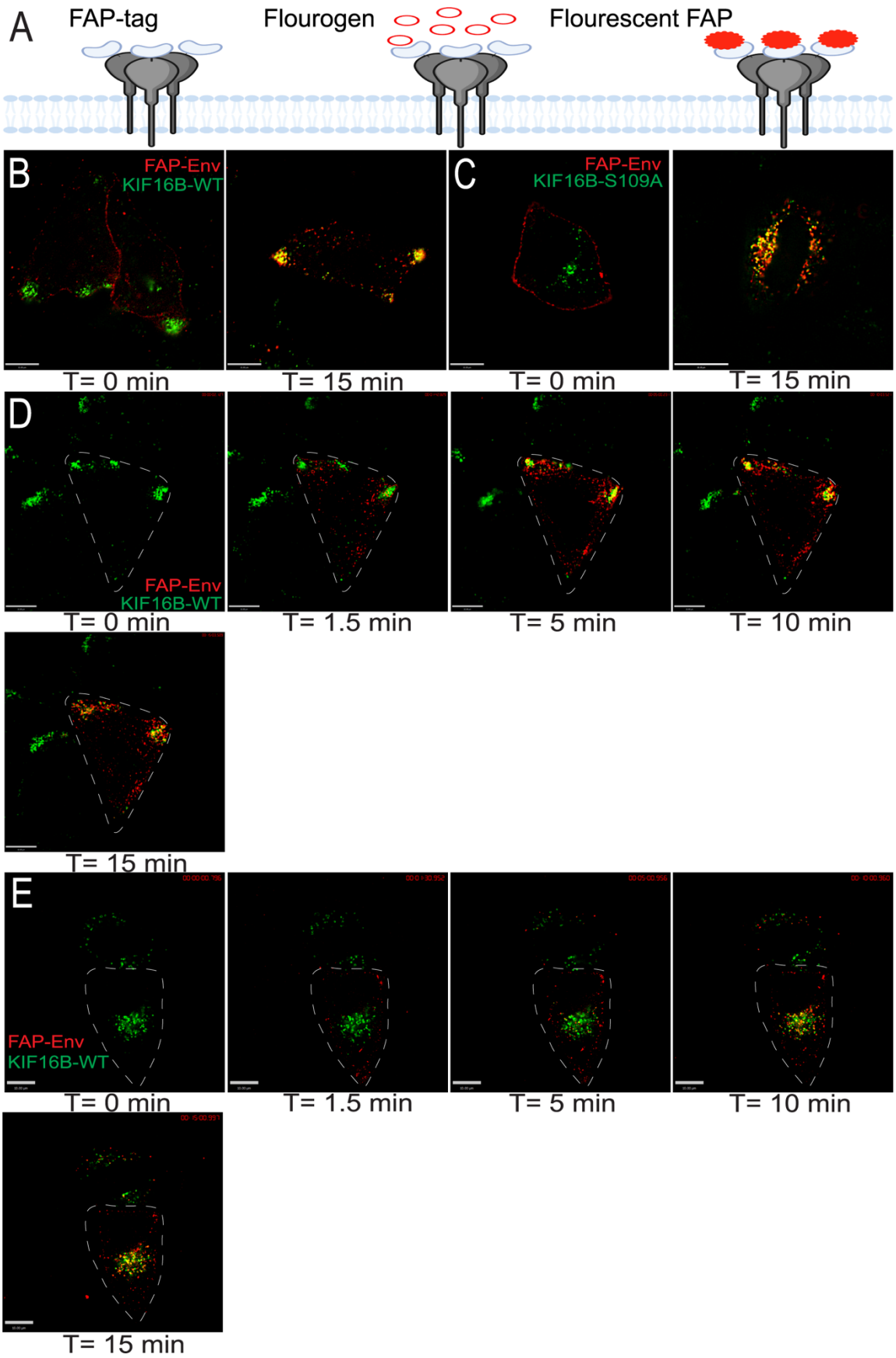
A
FAP-tag
Flourogen
Flourescent FAP
B
FAP-Env
KIF16B-WT
T= 0 min
T= 15 min
C
FAP-Env
KIF16B-S109A
T= 0 min
T= 15 min
D
FAP-Env
KIF16B-WT
T= 0 min
T= 1.5 min
T= 5 min
T= 10 min
T= 15 min
E
FAP-Env
KIF16B-WT
T= 0 min
T= 1.5 min
T= 5 min
T= 10 min
T= 15 min

760

761 **FIG 3** Env on the plasma membrane is endocytosed into KIF16B positive compartments.
762 (A) FAP-Env construct shown as cartoon diagram. The FAP-tag was placed on the V2
763 loop of the Env ectodomain. This tag is inherently non-fluorescent on the far left. In the
764 middle, the addition of a non-permeable dye is shown. On the far right, the FAP-tag
765 becomes fluorescent, allowing visualization of a pulsed population of surface Env. (B)
766 FAP-Env coexpressed with KIF16B- WT-YFP in HeLa cells. Left image, FAP-Env is pulse
767 labeled for 10 min on ice. Cells were then fixed and imaged immediately to show
768 population of Env on plasma membrane surface. Right image, cells were pulsed as before
769 and then were returned to 37°C incubator for 15 min to allow internalization, then fixed
770 and imaged to show FAP-Env endocytosed into KIF16B-positive compartments. (C) FAP-
771 Env construct co-expressed with KIF16B-S109A-YFP in HeLa cells. Left image is FAP-
772 Env collecting on surface as before. Right image is cell shown after 15 min of
773 internalization as before. (D, E) Frames from live cell imaging video of FAP-Env
774 coexpressed with KIF16B-WT-YFP and KIF16B-S109A-YFP, respectively. Time T= 0 min
775 shows cell before pulse. Subsequent time points show FAP-Env collecting in KIF16B-
776 positive compartments. Dotted lines are a visual aid to represent periphery of cell body.
777 Bars represent 10 μm.

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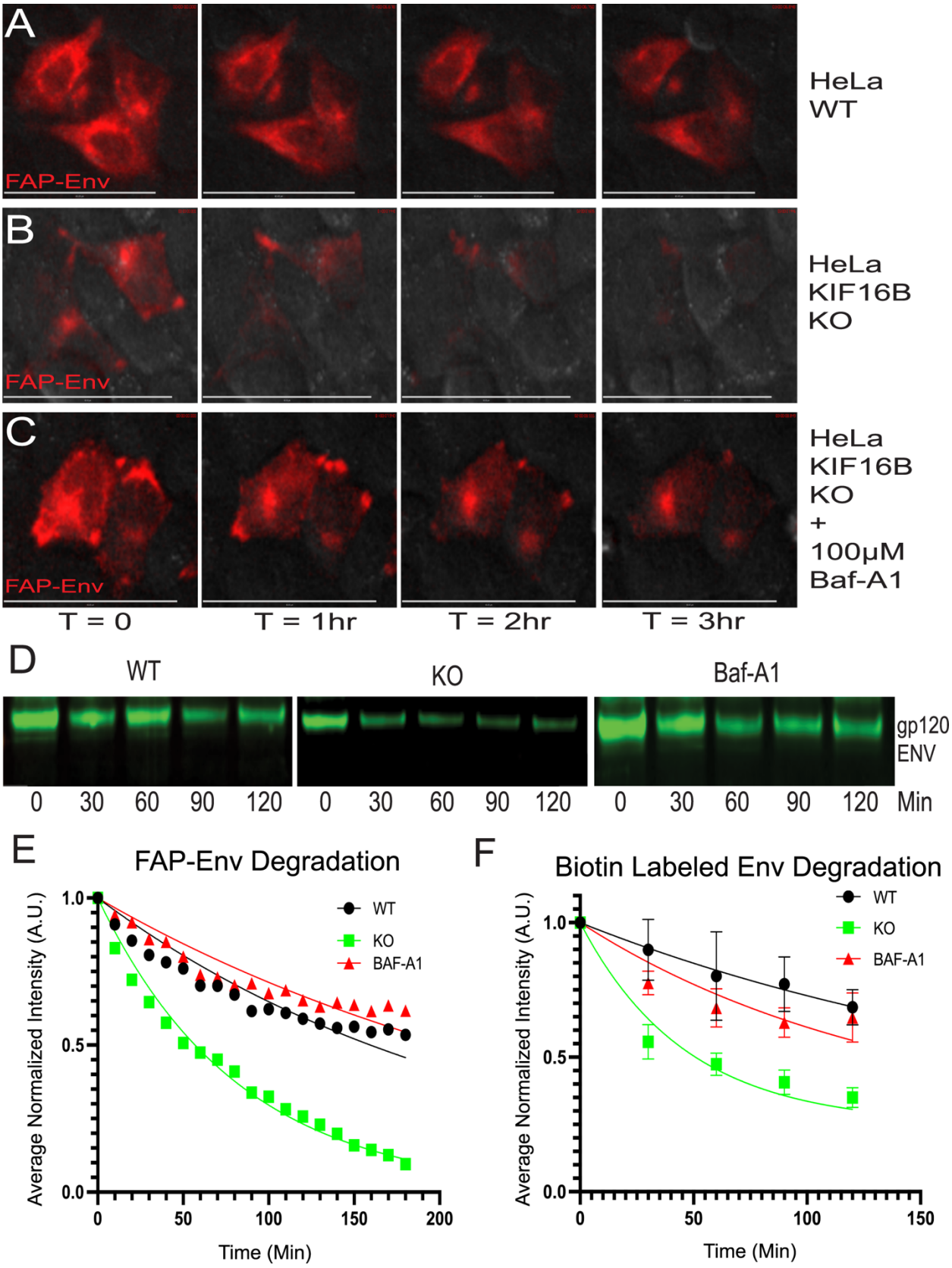
A
FAP-Env
HeLa
WT
B
FAP-Env
HeLa
KIF16B
KO
C
FAP-Env
HeLa
KIF16B
KO
+
100μM
Baf-A1
T = 0
T = 1hr
T = 2hr
T = 3hr
D
WT
KO
Baf-A1
gp120
ENV
0 30 60 90 120 0 30 60 90 120 0 30 60 90 120 Min
E
FAP-Env Degradation
Average Normalized Intensity (A.U.)
1.0
0.5
0.0
0 50 100 150 200
Time (Min)
WT
KO
BAF-A1
F
Biotin Labeled Env Degradation
Average Normalized Intensity (A.U.)
1.0
0.5
0.0
0 50 100 150
Time (Min)
WT
KO
BAF-A1

783

784 **FIG 4** KIF16B KO leads to enhanced degradation of endocytosed Env in the lysosome.
785 (A, B, C) Frames from live cell movies for Fap-Env expressed in (A) WT HeLa cells with
786 vehicle DMSO; (B) KIF16B KO cells with DMSO; or (C) KIF16B KO cells with 100μM Baf-
787 A1. Representative frames are shown for each hour over the time course. (B) Western
788 blots shown are biotinylated Env from surface-labeled cells, with lysates chased over the
789 time course, pulled down with streptavidin beads, and blotted for Env. Labels indicate
790 WT, KIF16B KO, and KIF16B KO + Baf-A1 HeLa cells. (C) Quantification of signal
791 intensity of each time point over the FAP-Env imaging experiment normalized to first
792 frame. (D) Quantification of the Western blot signal for each time point for biotin-
793 streptavidin pulse-chase experiment. Error bars represent SD from 5 independent
794 experiments. Fit curves for both C and D were generated using the Prism 9 non-linear
795 regression one-phase decay algorithm. Bars represent 60 μm.

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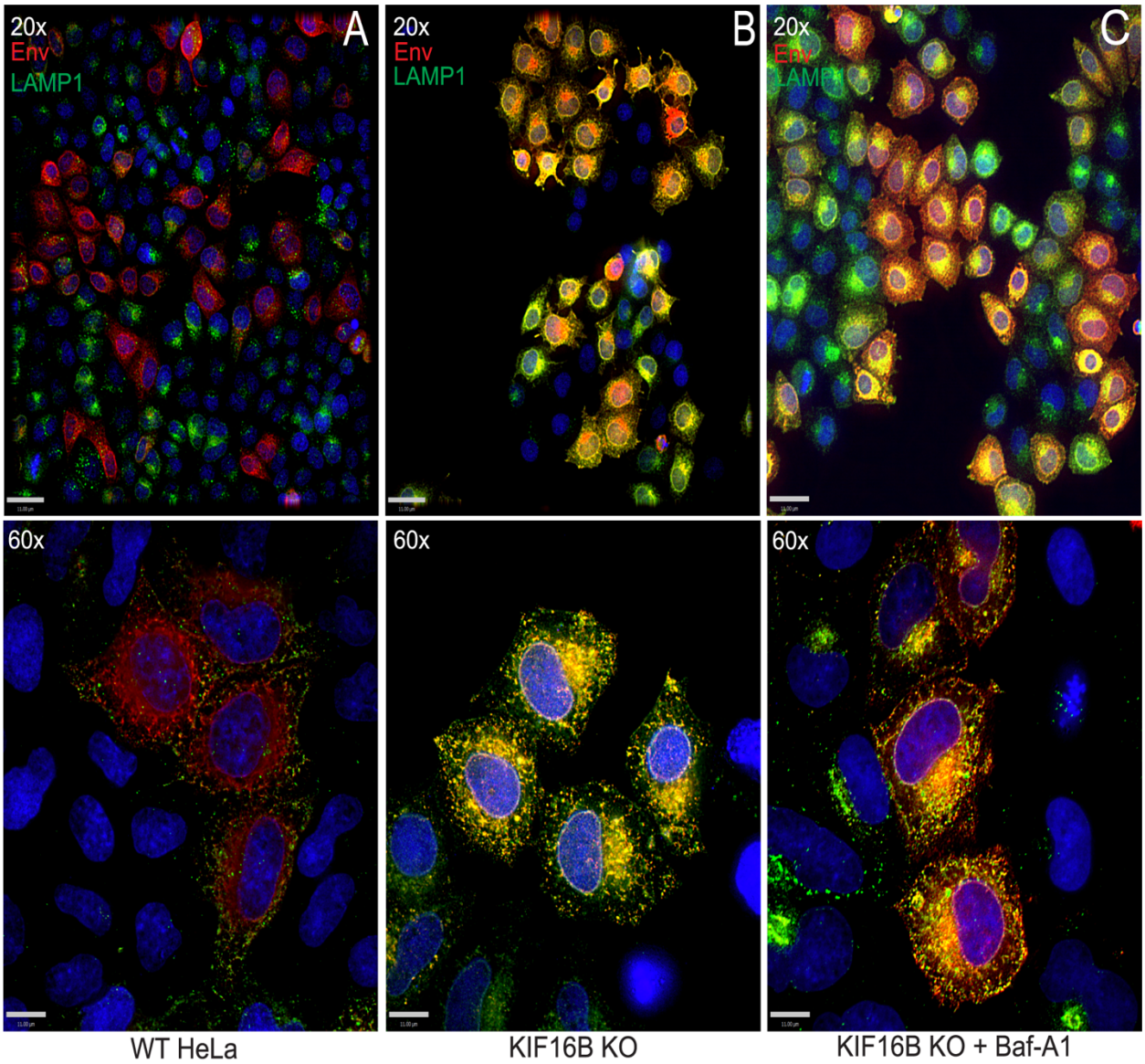
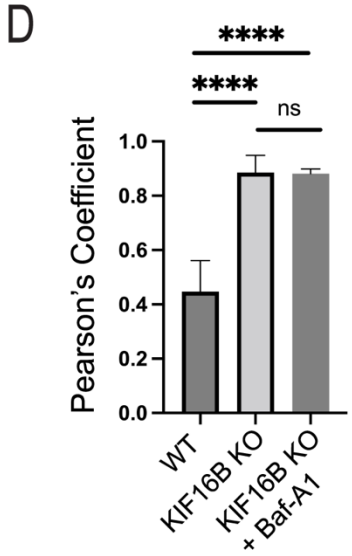

20x
Env
LAMP1
A
20x
Env
LAMP1
B
20x
Env
LAMP1
C
60x
60x
60x
WT HeLa
KIF16B KO
KIF16B KO + Baf-A1


D
****
****
ns
Pearson's Coefficient
1.0
0.8
0.6
0.4
0.2
0.0
WT
KIF16B KO
KIF16B KO + Baf-A1

805

806 **FIG 5** KIF16B KO shunts Env to lysosomal compartments. (A) HIV-1 NL4-3 Env
807 expressed in WT HeLa cells. Top image 20x magnification, bottom image 60x. (B) NL4.3
808 expressed in KIF16B KO HeLa cells. Top image 20x magnification, bottom image 60x.
809 (C) NL4-3 expressed in KIF16B KO HeLa cells with Baf-A1 added for 3 hours prior to
810 imaging. For all imaging, cells were fixed and immunolabeled for Env with 2G12 antibody
811 and for endogenous LAMP1 with H4A3 antibody. (D) Pearson's correlation coefficient
812 showing the degree of colocalization between Env and LAMP1 positive compartments
813 between WT, KIF16B KO cells, and WT cells with Baf-A1 in A, B, and C, respectively.
814 Pearson's correlation coefficient equation using Volocity 6.5.1 software after manual
815 thresholding. Unpaired t-test performed on Prism 9 to get significance values for
816 difference in colocalization. ****, $P < 0.0001$; *, $P = 0.0155$. Bars represent 11μm.

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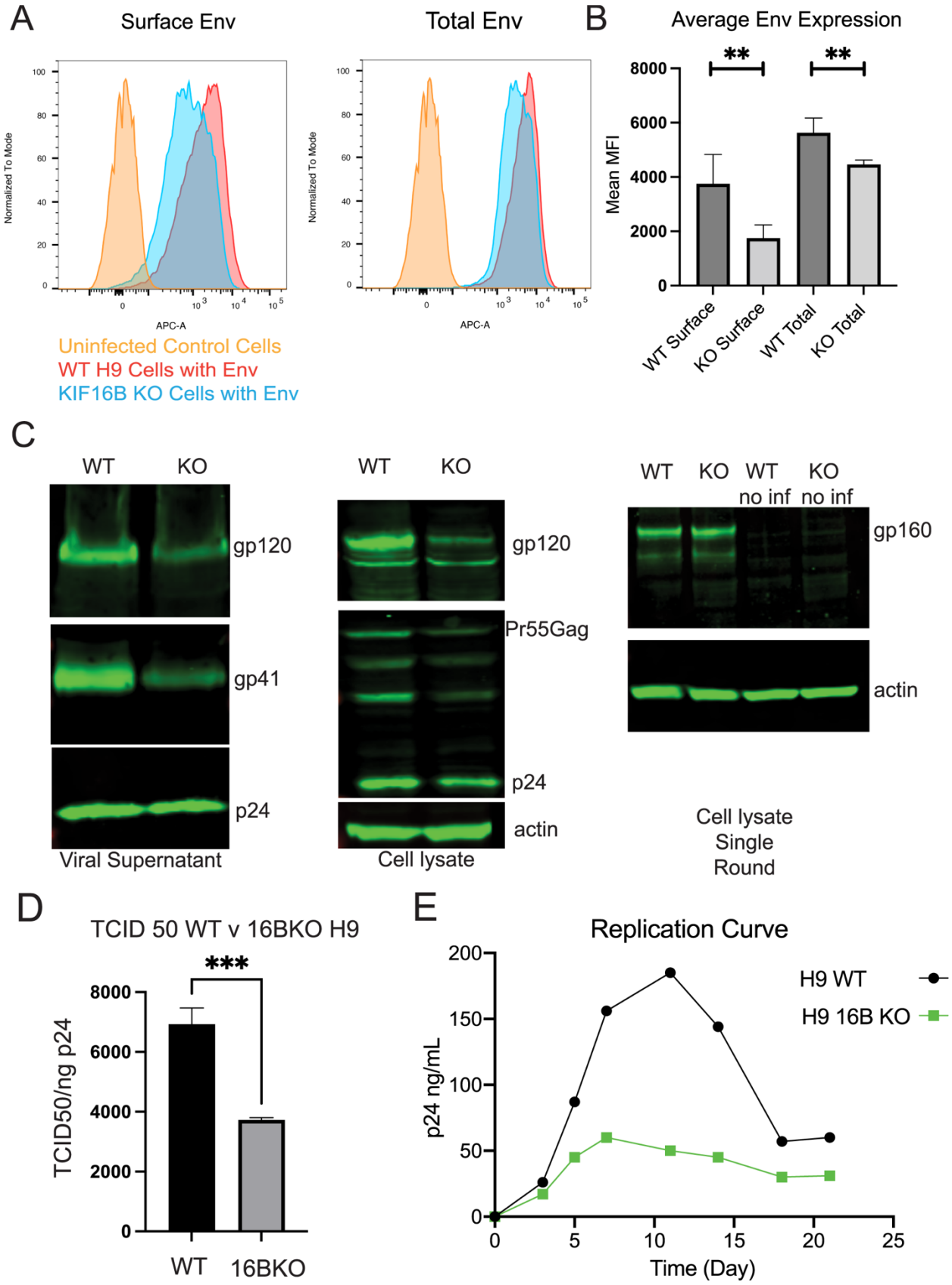
A
Surface Env
Total Env
Normalized To Mode
APC-A
Uninfected Control Cells
WT H9 Cells with Env
KIF16B KO Cells with Env
B
Average Env Expression
Mean MFI
**
**
WT Surface
KO Surface
WT Total
KO Total
C
WT
KO
gp120
gp41
p24
Viral Supernatant
WT
KO
gp120
Pr55Gag
p24
actin
Cell lysate
WT
KO
WT no inf
KO no inf
gp160
actin
Cell lysate Single Round
D
TCID 50 WT v 16BKO H9
***
TCID50/ng p24
WT
16BKO
E
Replication Curve
H9 WT
H9 16B KO
p24 ng/mL
Time (Day)

827

828 **FIG 6** KIF16B depletion leads to reduction of Env on the cell surface, particle
829 incorporation, infectivity, and viral replication. (A) Flow plots for cell surface staining (non-
830 permeabilized) and total Env content (permeabilized) in WT H9 and KIF16B KO H9 cells.
831 Cells were fixed and stained for Env using anti-gp120 2G12 antibody. (B) Quantification
832 of flow cytometry mean fluorescent intensity of Env expression on surface and total cell
833 averaged across 5 independent experiments. Unpaired t-test performed on Prism 9 to get
834 significance values between surface vs total. **, $P < 0.005$. (C) WT and KIF16B H9 cells
835 were infected with VSV-G-pseudotyped NL4-3. Shown are Western blots of Env and Gag
836 content for particles in viral supernatant (left) and cell lysates (middle) collected on day 5
837 following infection. Note that loading was normalized for p24 content (supernatants) or
838 for total cellular protein (cell lysates). Rightmost blot, single-round infection of WT and
839 KIF16B KO H9 cells infected with VSV-G-pseudotyped NL4-3 at an MOI of 0.5 and
840 harvested for cell lysate analysis after 24 hours. Uninfected WT and KO cell lysates were
841 run on the same blot as controls. Blotting was performed with 2G12 antibody against HIV-
842 1 gp160 and ACTN05 (Invitrogen) against actin as a loading control. (D) Infectivity of viral
843 particles released from WT or KIF16B KO H9 cells from 3 independent experiments
844 evaluated using TZM-bl indicator cells. (E) WT (black line) and KIF16B KO (green line)
845 were infected with VSV-G-pseudotyped NL4-3 virus. Viral growth was monitored over
846 time by detection of p24 in the culture supernatant.

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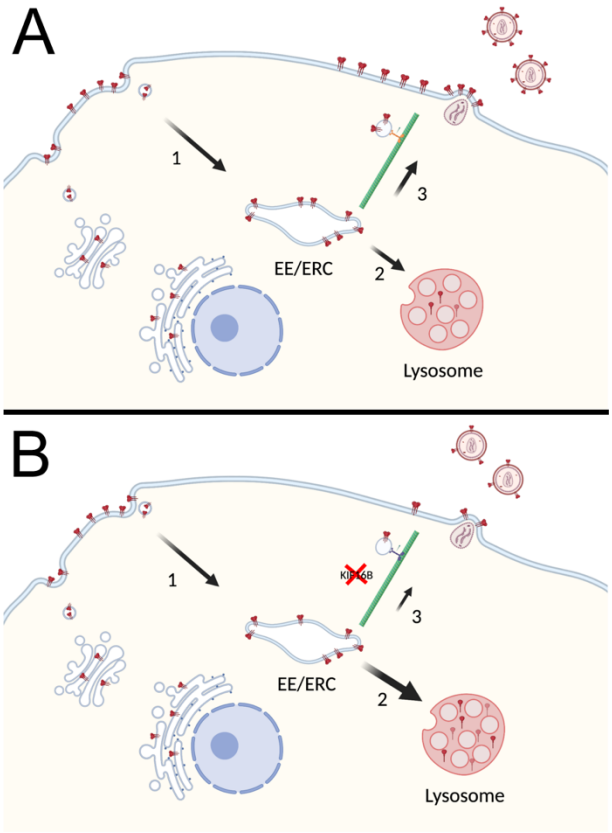

FIG 7 Model of Env Trafficking with (A) and without (B) KIF16B. (A) In a wild-type cell, Env is produced in the rough endoplasmic reticulum and follows the secretory pathway to the cell surface. Env is then endocytosed into early endosomes (path #1 in the figure), which later may sort to the endosomal recycling compartment (ERC) for recycling. This compartment is depicted as “EE/ERC”. A fraction of Env in EEs can progress to fuse with lysosomes, leading to lysosomal degradation in path #2. In path #3, Env is directed in an anterograde manner to the PM, where it is available for incorporation into viral particles. Path #3 is the relevant pathway that is partially dependent upon KIF16B. (B) In a cell with KIF16B KO, a diminished fraction of Env is delivered to the PM from the EE/ERC via path #3. This results in an increase in the amount of Env shunted to the lysosome (path #2,

862 illustrated as broader arrow) and enhanced degradation of Env. The change in the
863 distribution of endocytosed Env is shown graphically as fewer trimers on the budding and
864 mature virion, as well as fewer trimers on the PM and more trimers in the lysosome. Figure
865 created with BioRender.com.

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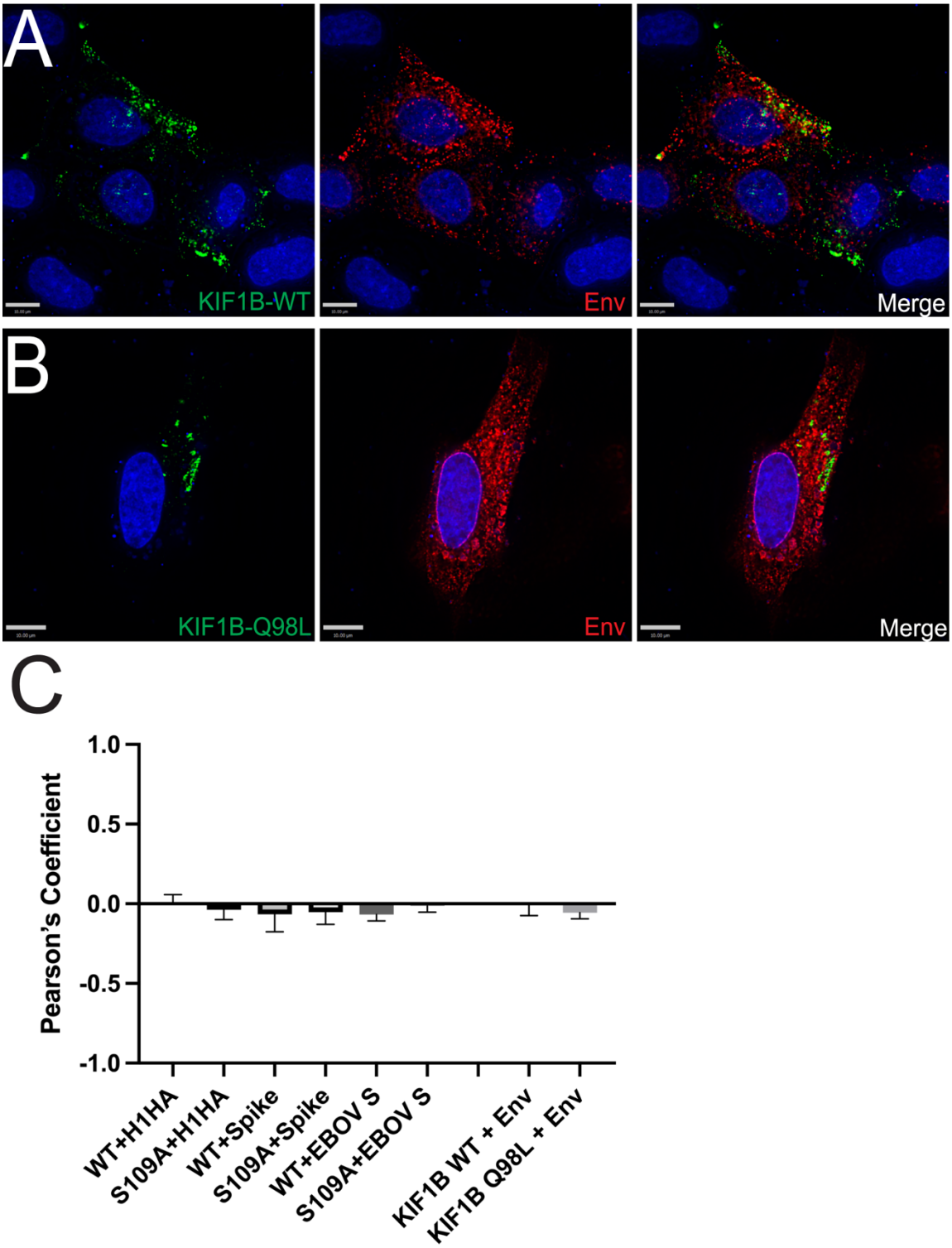
874 **SUPPLEMENTAL FIGURES**



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877 **FIG S1** (A, B) Coexpression of KIF1B WT and KIF1B Q98L (dominant-negative) with HIV-
878 1 Env. Cells were fixed and immunostained with 2G12 antibody specific to the Env
879 glycoprotein. Green, KIF1B; red, Env; rightmost image, overlay. (C) Degree of
880 colocalization for all viral glycoproteins included in Fig 2 with KIF16B (left six bars); and
881 colocalization of Env with KIF1B (rightmost two bars), measured by Pearson's correlation
882 coefficient equation using Volocity 6.5.1 software after thresholding. Results are shown
883 as means ± SD from a total of 5 representative images. Bars represent 10 μm.

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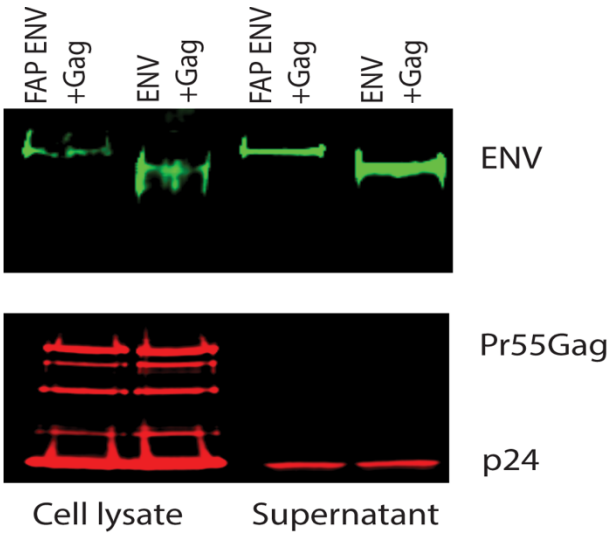

FIG S2 FAP-tagged Env or WT JR-FL Env were co-expressed with Env-deficient provirus. Western blot shows Env in cell lysates and on pelleted particles from supernatants. Env was blotted with 2G12 and Gag detected with CA183.

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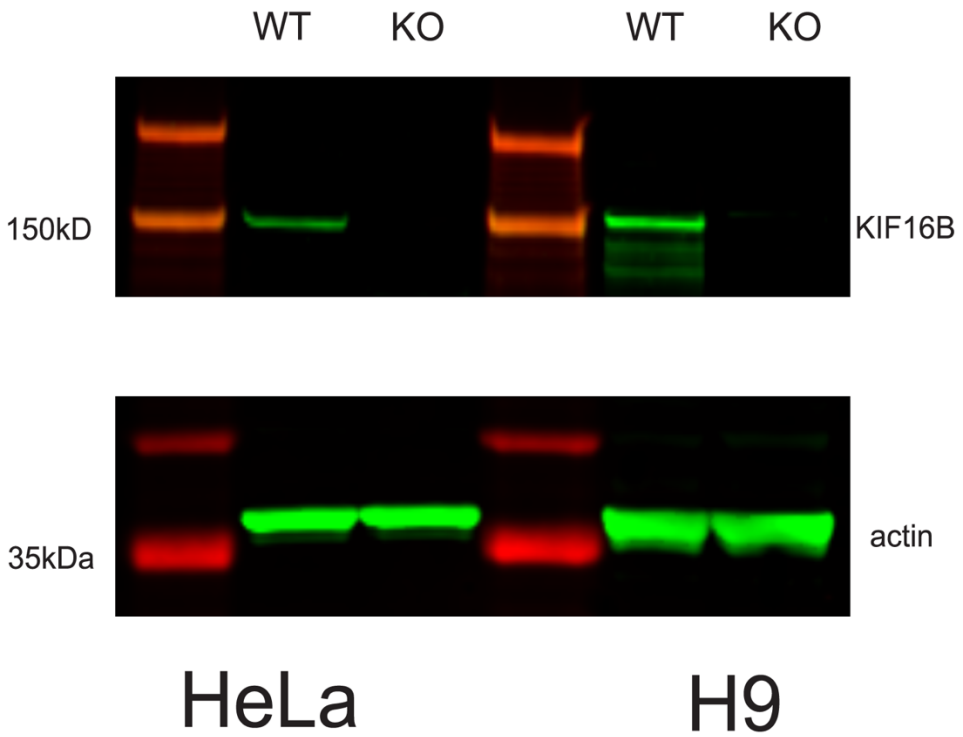
WT
KO
WT
KO
150kD
KIF16B
35kDa
actin
HeLa
H9

B

Status
Succeeded
Guide Target
CTCACGGATAAGTTTGACGT
PAM Sequence
TGG
Indel %
91
Model Fit (R^2)
0.91
Knockout-Score
76
EDITED SAMPLE 150 TO 215 BP
CONTROL SAMPLE 149 TO 214 BP

915 **FIG S3** (A) Representative blots of WT and KIF16B KO HeLa (left) and H9 (right) cell
916 lines blotted for KIF16B and actin loading control showing complete protein knock out in
917 both cell lines. (B) Sequence of genomic DNA from HeLa cells showing CRISPR-Cas9
918 indel. Dotted line indicates cut site of CRISPR-Cas9 complex. Edited sample sequence
919 on top for KIF16B KO cells, the control sample on bottom was the reference sequence
920 from WT cells that were the same cells used to make the knockout clones. Sequence
921 analyzed by ICE analysis via the Synthego software application.

922

923 **SUPPLEMENTAL VIDEO LEGENDS**

924 **Supplemental Video 1** FAP-Env is endocytosed into WT KIF16B positive compartments.
925 FAP-Env construct was co-expressed with WT KIF16B-YFP in HeLa cells. The next day,
926 cells were imaged in a live cell chamber at 37°F with 5% CO_2. Impermeable FAP reagent
927 was added to the cell media at 1.5 minutes and is seen upon addition. Over the course
928 of 15 minutes, this surface Env is endocytosed readily into KIF16B positive
929 compartments. Red, FAP-Env. Green, KIF16B. Bar is 10μm.

930 **Supplemental Video 2** FAP-Env is endocytosed into S109A dominant-negative KIF16B
931 positive compartments. FAP-Env construct was co-expressed with S109A-KIF16B-YFP
932 in HeLa cells. The next day, cells were imaged in a live cell chamber at 37°F with 5%
933 CO_2. Impermeable FAP reagent was added to the cell media at 1.5 minutes and is seen
934 upon addition. Over the course of 15 minutes, Env from the surface is endocytosed and
935 transverses the cytoplasm into S109A-KIF16B positive compartments near the nucleus.
936 Red, FAP-Env. Green, KIF16B. Bar is 10μm.

937 **Supplemental Video 3** KIF16B knockout in HeLa cells enhances FAP-Env degradation,
938 which is recovered by lysosomal inhibitor BAF-A1. (A, B, C) FAP-Env is expressed in (A)
939 WT HeLa cells with vehicle DMSO (B) KIF16B KO HeLa cells with DMSO and (C) KIF16B
940 KO plus 100μM BAF-A1 cells (DMSO or BAF-A1 is placed on cells 3 hours before
941 imaging) overnight prior to imaging. Live cell imaging performed at 37°F with 5% CO_2.
942 Impermeable FAP reagent was added to the cell media immediately prior to imaging.
943 Time course of experiment is shown in upper-right as 3 hours. Red, FAP-Env. Bars are
944 60μm.