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The tumour suppressor APC promotes HIV-1 assembly via interaction with Gag precursor protein

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Diverse cellular proteins and RNAs are tightly regulated in their subcellular localization to exert their local function. Here we report that the tumour suppressor adenomatous polyposis coli protein (APC) directs the localization and assembly of human immunodeficiency virus (HIV)-1 Gag polyprotein at distinct membrane components to enable the efficient production and spread of infectious viral particles. A proteomic analysis and subsequent biomolecular interaction assay reveals that the carboxyl terminus of APC interacts with the matrix region of Gag. Ectopic expression of APC, but not its familial adenomatous polyposis-related truncation mutant, prominently enhances HIV-1 production. Conversely, the depletion of APC leads to a significant decrease in membrane targeting of viral components, resulting in the severe loss of production of infectious virions. Furthermore, APC promotes the directional assembly of viral components at virological synapses, thereby facilitating cell-to-cell viral transmission. These findings reveal an unexpected role of APC in the directional spread of HIV-1.

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Human immunodeficiency virus (HIV)-1 is the causative agent of acquired immunodeficiency syndrome (AIDS) and has evolved to invade the complex human immune system and utilize the host machinery for the propagation of progeny virus[1,2]. It is well known that the orchestrated participation of viral components and host factors is required for persistent and efficient virus replication. Previous comprehensive genome-wide analyses have revealed hundreds of host proteins to be related to HIV-1 replication[3–5]. However, their functional relevance and the nature of their contribution to HIV-1 propagation in the context of diverse cellular functions, such as cell polarity and cell-to-cell communication remain largely unknown.

In the late stage of the HIV-1 replication cycle, the intracellular trafficking of the viral structural protein Gag (also known as $Pr55^{Gag}$) and viral genomic RNA (vRNA) to the plasma membrane (PM) is a crucial step for the efficient production of infectious virions. The Gag precursor is composed of four functional domains: matrix (MA), capsid (CA, also called p24), nucleocapsid (NC) and p6, and two spacer sequences (Sp2 and Sp1). The MA domain is responsible for the PM targeting of Gag polyprotein. In fact, the hydrophobic myristate anchor at the N terminus of MA can insert into the hydrophobic core of PM. Furthermore, a cationic patch of basic residues on MA forms electrostatic interactions with anionic membrane lipids such as phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$)[6–10]. At the PM, both CA-mediated Gag multimerization and NC-mediated vRNA incorporation can drive viral assembly and production of nascent virions[11]. Live cell imaging analysis has suggested also that Gag could be required for stable association of vRNA with the PM[12,13]. However, it is not fully understood how these viral assembly processes are further regulated during and after Gag–PM binding is completed.

Cellular polarity generates dynamic and spatial patterns both inside and outside of the cell. In terms of virus infection, cell polarity creates a more efficient and dynamic assembly process[14]. Indeed, in polarized cells, viral components are dynamically transported to defined domains and/or structures on the PM, including membrane nanotubes, filopodial bridges or uropods, for efficient assembly and budding[15,16]. These specific membrane structures are generally enriched with actin filaments and can provide the topological spaces for not only the formation of infectious viral particles, but also their deliberate spread with spatial orientation. The most extensively studied among them is the virological synapse (VS), in which the nascent virus is directly passed between two apposed PMs from the infected cell to the neighbouring uninfected cell. It is widely believed that cell-to-cell viral transfer is a major mode of infection in lymphoid tissues[17] and is 10^2- to 10^3-fold more efficient for spreading HIV-1 than cell-free infection[18]. Hence, developing an understanding of the host factors that contribute to the targeting of HIV-1 components to the specific site of virus assembly such as the VS might provide an important clue to developing a new anti-retroviral strategy.

In our present study, we demonstrate that the tumour suppressor adenomatous polyposis coli protein (APC) directly binds HIV-1 Gag and regulates the intracellular localization of the viral components for directional HIV-1 assembly. Consequently, APC was found to enhance the VS-mediated cell-to-cell transmission of HIV-1. These findings uncover a previously uncharacterized function of APC in HIV-1 replication and thus provide important new insights into the molecular mechanisms underlying HIV-1–host cell interactions.

Results

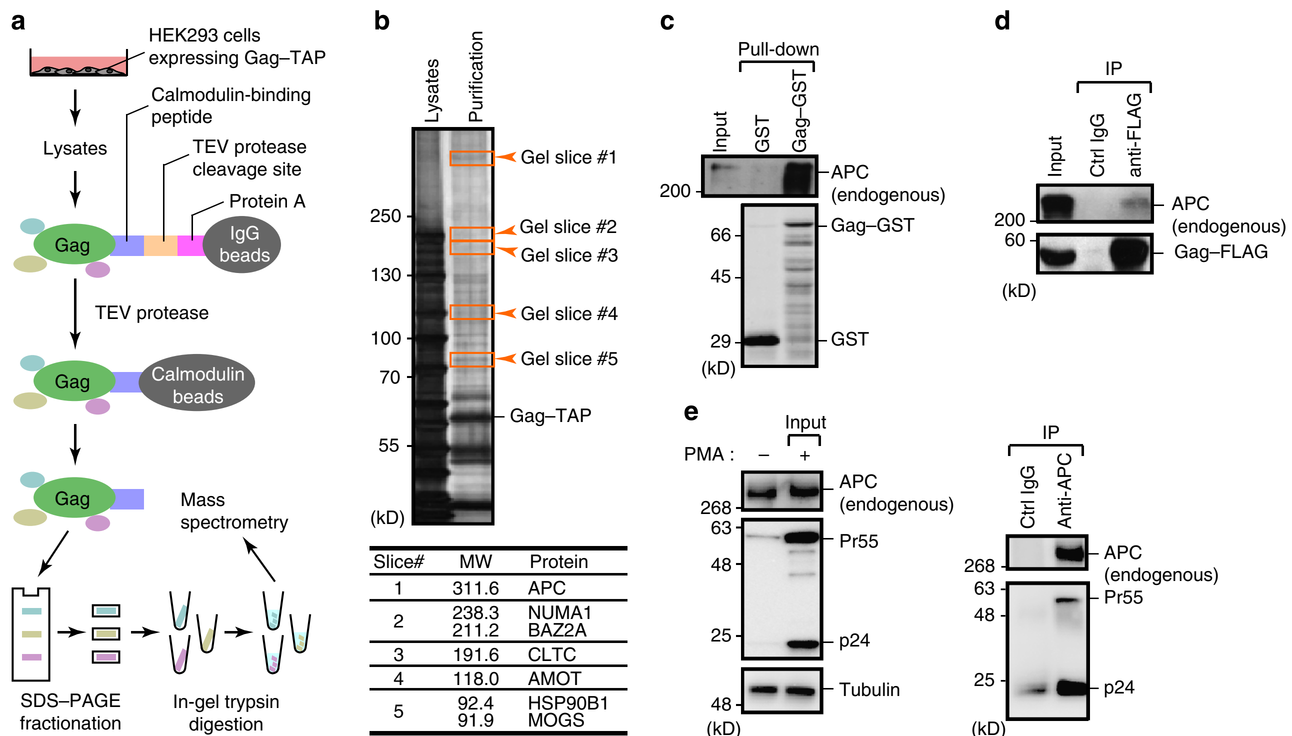
Identification of APC as a HIV-1 Gag-interacting protein. Gag is a major component of HIV-1 and plays a crucial role in its assembly. To better understand the host proteins that promote this assembly, we used the tandem affinity purification (TAP) approach[19] to identify HIV-1 Gag-interacting protein(s). We purified the Gag-associated complex from the cell lysates of HEK293 cells expressing HIV-1 Gag fused to a C-terminal TAP tag, which contains an IgG-binding motif and calmodulin-binding motif separated by a tobacco etch virus (TEV) protease cleavage site (Fig. 1a). Gag–TAP-bound proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE) and visualized by silver staining. Specific bands were then excised and in-gel digested with trypsin. Subsequent mass spectrometry analysis identified the APC protein as a candidate of Gag-binding proteins (Fig. 1b; Supplementary Table 1). These also included angiomotin (AMOT), which is a previously reported Gag-binding factor[20], indicating the validity of our current experimental approach. Glutathione-S-transferase (GST) pull-down and immunoprecipitation analysis further revealed the interaction between Gag and endogenous APC in cells (Fig. 1c,d). Furthermore, this interaction was also found in HIV-1-infected T cells, where Gag protein was immunoprecipated with endogenous APC (Fig. 1e).

We next attempted to determine the binding regions on both proteins that facilitate the Gag–APC interaction. For this purpose we performed *in vitro* quantitative protein–protein interaction analysis (AlphaScreen; amplified luminescent proximity homogeneous assay)[21,22] using recombinant Gag–GST and APC–FLAG proteins (Fig. 2a). In our assay, we used seven truncated constructs derived from APC, since the full-length APC protein was quite difficult to synthesize using a wheat cell-free system due to its larger size (>300 kD). Consequently, our AlphaScreen analysis clearly demonstrated that C-terminal region of APC (APC-CT) could associate with Gag (Fig. 2b). Notably, two C terminally truncated APC mutants (1–1,309 and 1–1,462) found in familial adenomatous polyposis (FAP) patients[23,24], failed to interact with Gag (Fig. 2b). We next investigated the binding domain in Gag, which consists of four major subunits, MA, CA or p24, NC and p6. AlphaScreen analysis demonstrated that APC-CT could preferentially bind MA (Fig. 2c). Taken together, our findings suggested that the APC-CT interacts with the Gag MA domain.

APC facilitates HIV-1 particle production. To examine whether APC affects viral particle production through its interaction with Gag, we first transfected HEK293 cells with a HIV-1 molecular clone pNL4-3 (encoding full-length $HIV\text{-}1_{NL4\text{-}3}$ genome)[25], together with APC expression vectors. We found that wild-type (WT) APC, but not a C terminally truncated FAP–APC (1-1,309), significantly enhanced HIV-1 particle production (Fig. 3a). We performed parallel experiments using SW480 colon carcinoma cells expressing only a C terminally truncated form of APC protein endogenously[26], and found that the exogenous expression of WT APC resulted in the prominent increase in HIV-1 particle production (Fig. 3b). Interestingly, we found that APC-CT could not enhance, but rather reduced viral particle production (Fig. 3c), suggesting that while this region is important for Gag binding (Fig. 2b), it has a functionally dominant-negative effect on HIV-1 production.

We next addressed whether the effects of APC on HIV-1 assembly are due to APC's role in Wnt/β-catenin-dependent signalling. We found that the function of APC in relation to HIV-1 seems not to be caused by inhibition of Wnt/β-catenin signalling, since the overexpression of neither β-catenin nor dominant-negative T-cell transcription factor 4 (TCF4) affected the effects of APC on HIV-1 particle production (Supplementary Fig. 1a–d).

The MA region of Gag is essential for membrane binding of Gag and hence for HIV-1 assembly and budding[27]. Meanwhile,



Slice#	MW	Protein
1	311.6	APC
2	238.3 211.2	NUMA1 BAZ2A
3	191.6	CLTC
4	118.0	AMOT
5	92.4 91.9	HSP90B1 MOGS

Figure 1 | Identification of the tumour suppressor APC as a HIV-1 Gag-binding protein. (**a**,**b**) HEK293 cells expressing Gag fused with TAP tag (IgG-TEV-calmodulin-binding motif) were lysed and subjected to TAP-tag-based purification. (**a**) Proteins bound to Gag–TAP were isolated and detected by SDS-PAGE and silver staining. (**b**, top) Peptides derived from the excised gel slices (arrowheads) were analysed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (**b**, bottom). Detailed information on the proteins identified by mass spectrometry is provided in Supplementary Table 1. MW, molecular weight. (**c**) Extracts of HEK293 cells were subjected to GST pull-down analysis using either GST or Gag-GST followed by immunoblotting with anti-APC antibody. (**d**) HEK293 cells expressing Gag-FLAG were lysed and subjected to immunoprecipitation with anti-FLAG antibody followed by immunoblotting with anti-APC antibody. (**e**) ACH-2 cells stimulated with phorbol 12-myristate 13-acetate (PMA) were lysed and subjected to immunoprecipitation with anti-APC antibody followed by immunoblotting with anti-p24 antibody. Full images for all immunoblots are provided in Supplementary Fig. 4.

the addition of an N-terminal 10-amino-acid Fyn kinase sequence (Fyn(10)), which mediates protein–membrane binding via a triple acylation signal, is sufficient to rescue partially the budding of MA-deficient virus[28]. To assess the impact of the MA domain on the APC-dependent enhancement of virus particle production, we utilized HIV-1_{NL4-3} mutants harbouring either WT Gag or MA-deleted Gag with N-terminal Fyn(10), designated as Fyn(10)WT and Fyn(10)ΔMA, respectively (Fig. 3d), both of which have been shown to have assembly and budding capacity[28]. We observed that the production of virus particles from parental HIV-1 and Fyn(10)WT, but not its MA-deficient derivative Fyn(10)ΔMA, were promoted by APC (Fig. 3d). These results suggested that APC functionally interacts with the MA region of Gag to enhance HIV-1 particle production. Since APC has been found to play a crucial role in a wide variety of cellular processes, including the organization of cytoskeleton networks[29], we next investigated whether the function of APC in promoting HIV-1 particle production is dependent on cytoskeleton dynamics. Parallel analysis with several inhibitors targeting cytoskeletal organization revealed that the inhibition of actin polymerization by cytochalasin B and latrunculin B abrogated the function of APC in facilitating HIV-1 release (Fig. 3e). This suggested that actin dynamics may be a prerequisite for APC-mediated HIV-1 particle production.

Highly basic region in Gag MA is crucial for APC function. Previous studies have demonstrated that the highly basic region (HBR) within the Gag MA has an important role in the targeting of Gag to the specific microdomain of PM for virus assembly[30,31]. To investigate the requirement of these basic residues for the functional interaction with APC, we used a NL4-3 derivative whose basic residues in HBR were substituted by neutral residues (Fyn(10)-Gag(6A2T)-YFP)[30] or with another basic residue (Fyn(10)-Gag(RKswitch)-YFP; ref. 30; Fig. 4a). Despite the changes in HBR, these Gag constructs retained their membrane-binding ability due to the Fyn N-terminal sequence, and a substantial fraction of Gag was found to be present at the PM. Notably, we observed that the production of virus particles from cells infected with HIV-1_{NL4-3} harbouring Fyn(10)WT and RKswitch mutant, but not its 6A2T mutant, was enhanced by APC (Fig. 4a). Consistent with this, AlphaScreen analysis and immunofluorescence staining revealed that APC could interact and co-localize with Fyn(10)WT and RKswitch mutant, but not 6A2T mutant (Fig. 4b,c). These results together suggested that the cationic property on HBR of Gag MA is important for the APC-mediated regulation of HIV-1 assembly.

APC regulates the recruitment of Gag to the PM. We next addressed whether APC could modulate the intracellular dynamics of Gag using a C terminally green fluorescent protein (GFP)-fused Gag (Gag–GFP). Consistent with previous reports[32–34], three patterns of Gag localization were observed (Fig. 5a). We analysed for the subcellular localization of Gag–GFP

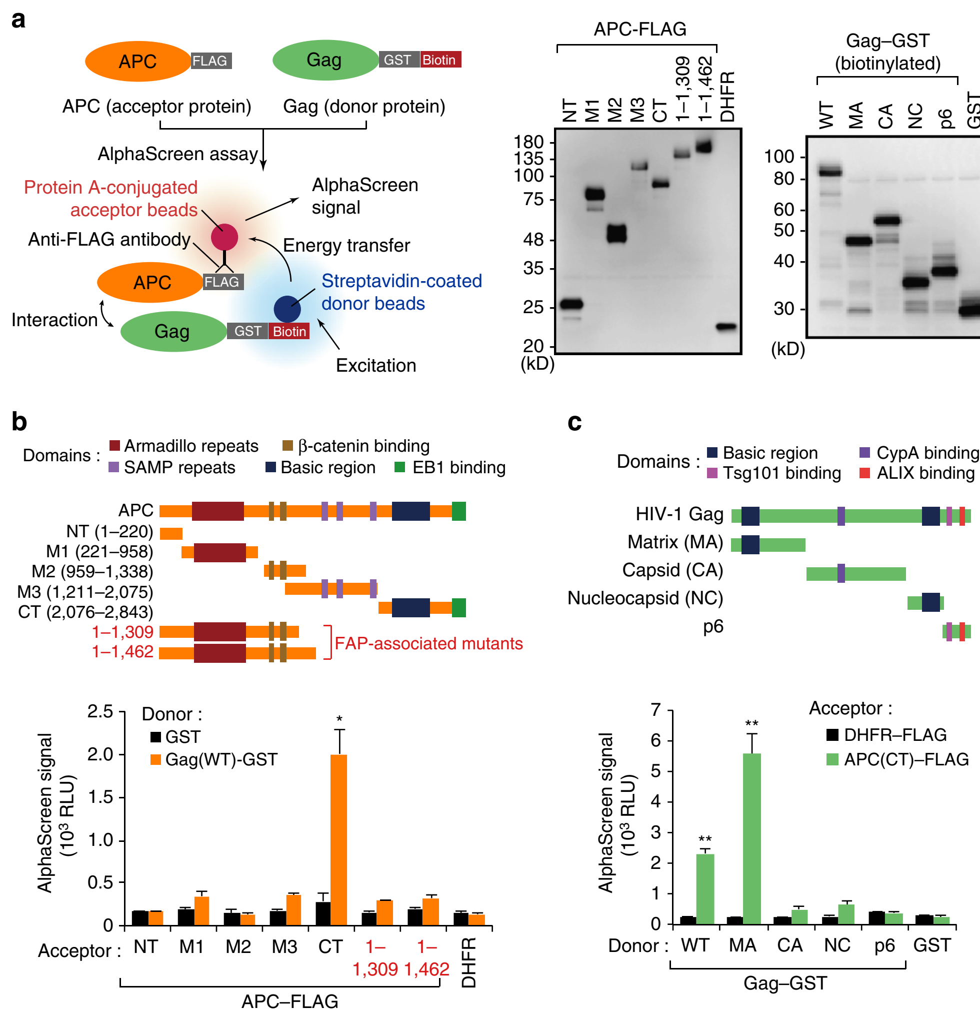


Figure 2 | The C-terminal region of APC interacts mainly with Gag MA. (**a**) Schematic representation of the amplified luminescent proximity homogenous assay (AlphaScreen) used to detect Gag–APC interactions. Gag-GST-biotin with donor beads and APC-FLAG with acceptor beads were mixed. If the two proteins can bind to each other, AlphaScreen signals were detected (left). All recombinant proteins used in the AlphaScreen assay were synthesized using a wheat germ cell-free system and detected by immunoblotting with anti-FLAG antibody and horse radish peroxidase-conjugated streptavidin (right). Full images for all immunoblots are provided in Supplementary Fig. 4. (**b**,**c**) AlphaScreen assays with recombinant APC truncated mutants (**b**) and Gag domains (**c**). FLAG-DHFR (dihydrofolate reductase) and GST were used as negative controls. All graphs are presented as a mean ± s.d. ($n = 3$). *$P < 0.05$, **$P < 0.01$, two-tailed unpaired t-test.

over 100 cells. At 8 h post transfection, Gag was distributed in a diffuse pattern throughout the cell irrespective of APC expression, whereas its localization was predominantly observed on the PM at 18 h in control cells. Notably, APC-depleted cells exhibited a decreased PM distribution of Gag at 18 h post transfection (Fig. 5a). Following membrane targeting, Gag forms high-order oligomers at the PM[35]. We thus performed direct measurements of Gag–Gag interaction in living cells using nano-bioluminescence resonance energy transfer (NanoBRET)-based protein–protein interaction analysis[36]. Our results demonstrated that APC-depleted cells exhibited less Gag–Gag interactions compared with control cells (Fig. 5b).

Our aforementioned results indicated that APC promotes the multimerization of Gag molecules as a likely result of the enhanced localization of Gag at the PM. To further evaluate the role of endogenous APC in HIV-1 particle production, HCT116 colon carcinoma cells harbouring full-length APC alleles were co-transfected with APC-specific short interfering RNA (siRNA) and HIV-1 molecular clone. The viral production from APC-depleted cells was significantly reduced compared with the cells transfected with control siRNA, while the total amount of $Pr55^{Gag}$ in cells was not altered upon the depletion of APC (Fig. 5c). Subsequently, we investigated the function of APC for multi-cycle HIV-1 replication in T cells. We generated Jurkat T cells expressing APC-specific short hairpin RNA (shRNA), which depleted the endogenous APC protein levels (Fig. 5d). Notably, APC-depleted T cells exhibited a lower level of HIV-1 replication than control T cells (Fig. 5d). This was also observed in primary $CD4^+$ T cells introduced with APC-shRNA as compared with control cells (Fig. 5e). Taken together, our findings suggest a crucial role of endogenous APC in efficient HIV-1 assembly and spread.

APC regulates the viral packaging of HIV-1 vRNA. The accumulation of vRNA at the site of viral assembly and subsequent vRNA packaging into virions are fundamental processes for the production of infectious HIV-1 particles. Several recent reports indicated that the vRNA localization largely depended on Gag association with PM[12,13]. We thus asked

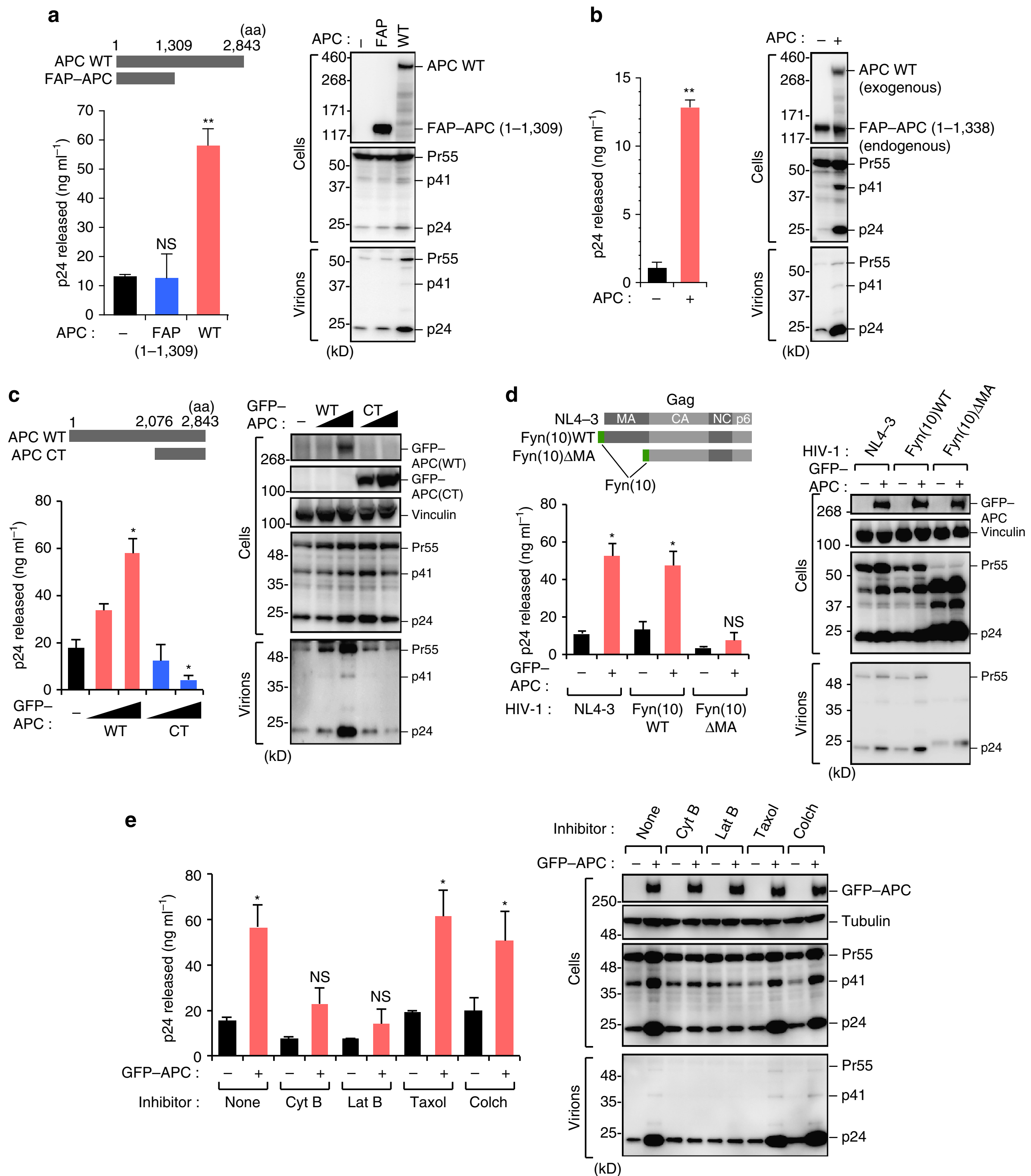


Figure 3 | APC facilitates HIV-1 particle production. (**a**,**b**) HEK293 (**a**) and SW480 (**b**) cells were co-transfected with pNL4-3 and plasmids encoding APC (WT or its FAP-related mutant). At 48 h after transfection, cells and supernatants were collected and analysed by immunoblotting using the indicated antibodies. The bar charts indicate the viral p24 antigen levels in the culture supernatants. (**c**) HEK293 cells were co-transfected with pNL4-3 and plasmids encoding APC (WT or its C-terminal domain, CT). At 48 h after transfection, culture supernatants and cell lysates were analysed by p24 enzyme-linked immunosorbent assay (ELISA) and immunoblotting, respectively. (**d**) HEK293 cells were transfected with vectors encoding GFP–APC and the indicated molecular clones encoding WT or MA-deleted Gag. At 48 h following transfection, culture supernatants and cell lysates were analysed by p24 ELISA and immunoblotting. (**e**) HEK293 cells were co-transfected with pNL4-3 and the indicated plasmids encoding APC. The indicated inhibitors were added to cells at 20 h before collecting. At 48 h following transfection, culture supernatants and cell lysates were analysed by p24 ELISA and immunoblotting. The final concentrations of inhibitors were as follows: Cyt B, Cytochalasin B (20 μM); Lat B, Latrunculin B (5 μM); Taxol (100 nM); Colch, Colchicine (50 μg ml^{-1}). All graphs are presented as a mean ± s.d. ($n = 3$). NS, not significant; *$P < 0.05$, **$P < 0.01$, two-tailed unpaired t-test. Full images for all immunoblots are provided in Supplementary Fig. 4.

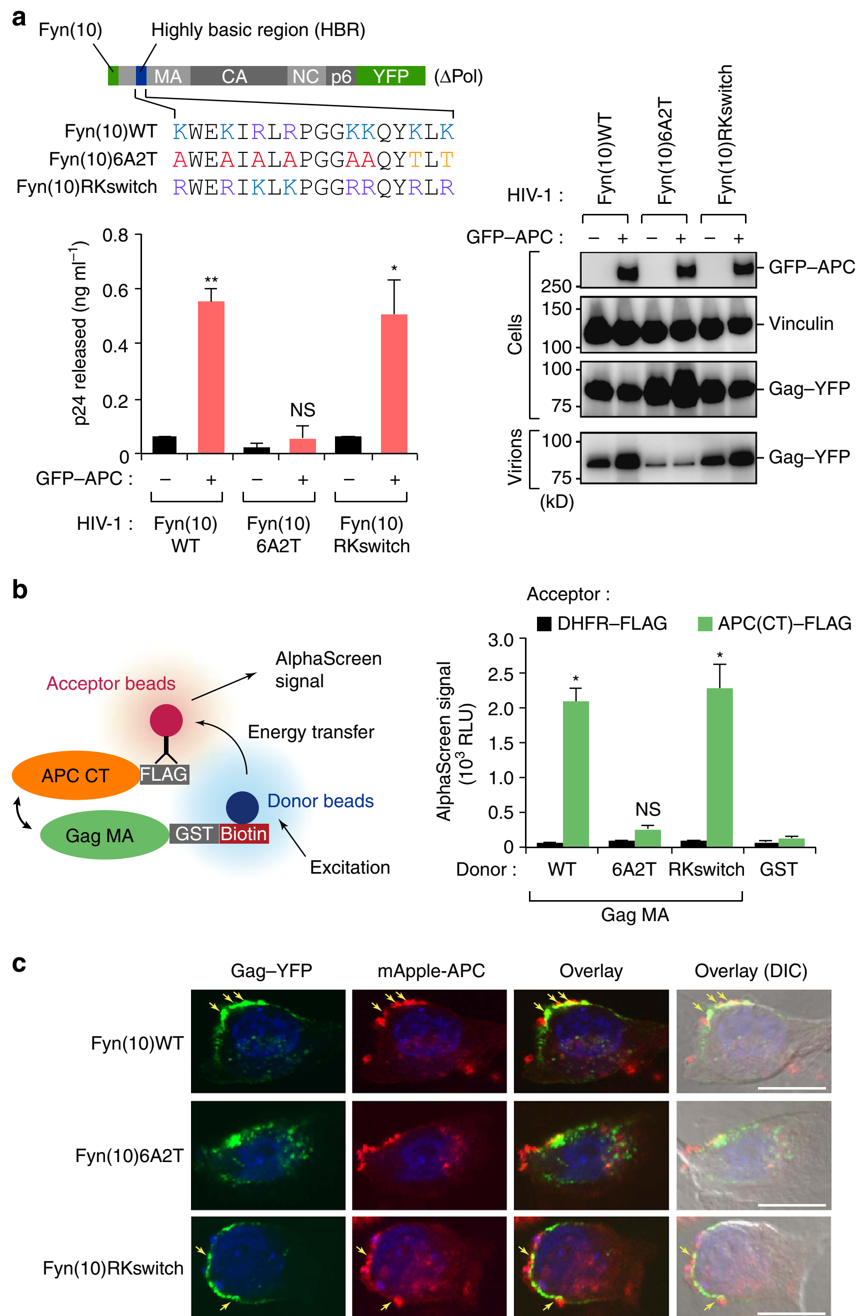


Figure 4 | The highly basic region in Gag MA is crucial for APC-mediated viral assembly. (**a**) Schematic representation of the pNL4-3-based derivative whose basic residues in Gag MA HBR were substituted by neutral residues (Fyn(10)-Gag(6A2T)-YFP) or with another basic residue (Fyn(10)-Gag(RKswitch)-YFP). HEK293 cells were transfected with these molecular clones and vectors encoding GFP–APC. At 48 h following transfection, culture supernatants and cell lysates were analysed by p24 enzyme-linked immunosorbent assay and immunoblotting. (**b**) AlphaScreen assays of recombinant APC-CT and MA HBR mutants. DHFR and GST were used as negative controls. (**c**) A549 cells were transfected with indicated molecular clones and together with mApple-APC expression vector. At 24 h following transfection, the cells were fixed and stained with 4,6-diamidino-2-phenylindole (nuclei, blue). Scale bar, 10 µm. The arrows indicate the co-localization of Gag and APC at the plasma membrane. All graphs are presented as a mean ± s.d. ($n=3$). *$P<0.05$, **$P<0.01$, two-tailed unpaired t-test. Full images for all immunoblots are provided in Supplementary Fig. 5.

whether APC can facilitate the PM localization and packaging of vRNA. The virions from cell supernatants were prepared and normalized to the Gag p24 levels. We then quantified the amounts of vRNA in the virions and measured their infectivity using TZM-bl reporter cells (Fig. 6a). Interestingly, the levels of virion-incorporated vRNA from APC-depleted cells were significantly reduced (Fig. 6b). Consistently, HIV-1 infectivity was also reduced in the virions from APC-depleted cells (Fig. 6c). To monitor the intracellular dynamics of vRNA in cells, we created a HIV-1 molecular clone carrying 24 repeats of the MS2-binding site (pNL4-3-pol-MS2x24) in the Pol region (Supplementary Fig. 2a). In this system, co-expression of an RNA-binding protein MS2 fused with GFP allows for the specific tagging of RNA containing MS2-binding sites in cells. As reported previously[12], solely expressed MS2–GFP proteins were mainly observed in the nucleus (Supplementary Fig. 2b).

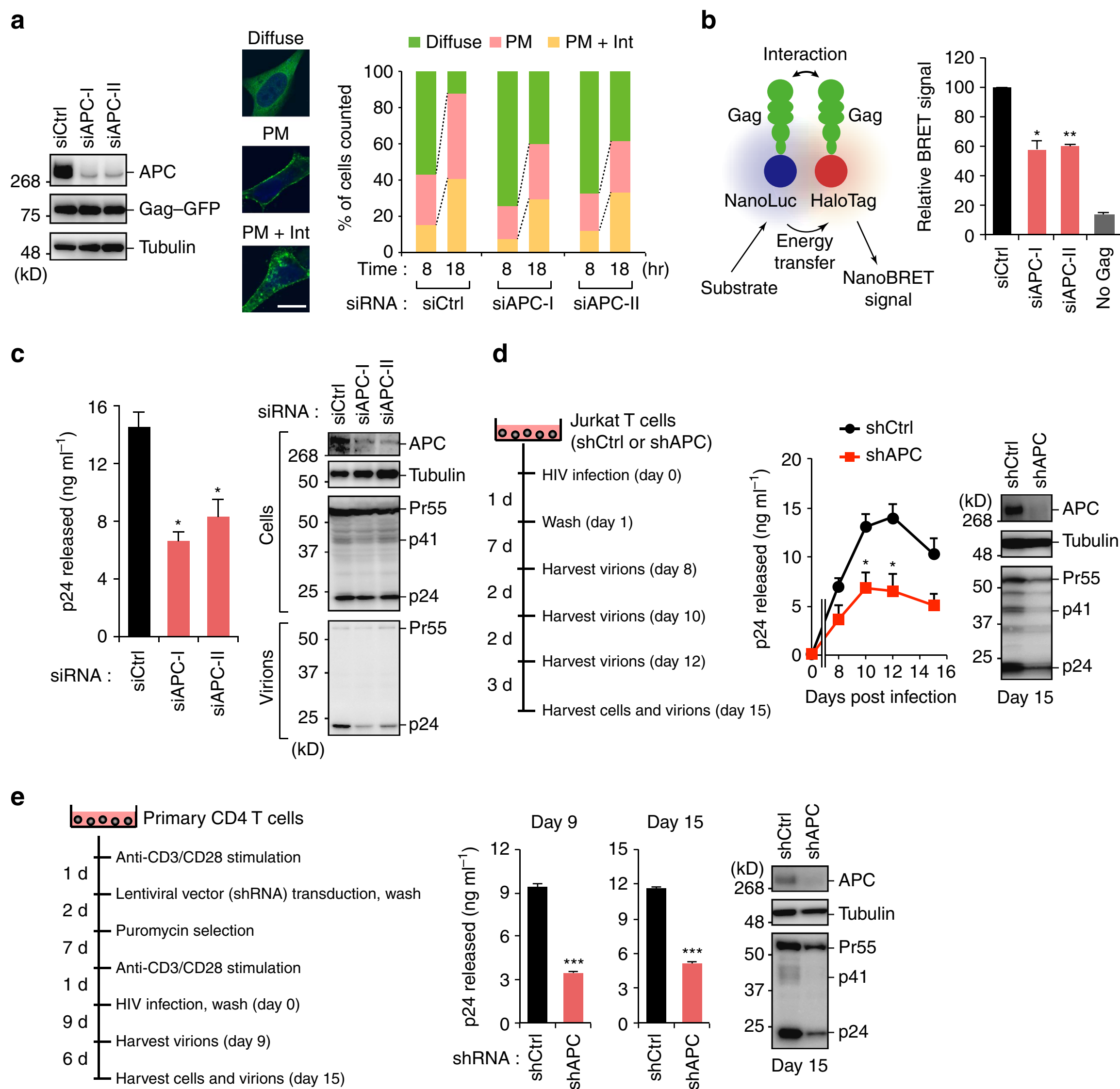


Figure 5 | APC regulates the recruitment of Gag to the PM. (**a**) HeLa cells were treated with control siRNA (siCtrl) or two different sequences of APC-targeted siRNAs (siAPC-I or -II) for 24 h before transfection with Gag–GFP expression vector. Blots show the detection of APC, tubulin and Gag–GFP by immunoblotting. At 8 and 18 h after transfection, over 100 cells were analysed for the subcellular localization of Gag–GFP, which was either strongly evident at the plasma membrane only (PM), at the PM and intracellular accumulations (PM + Int), or diffusely in the cytoplasm (diffuse). The data are percentages of the total cells. Scale bar, 10 μm. (**b**) HeLa cells were transduced with control siRNA (siCtrl) or two different sequences of APC-targeted siRNAs (siAPC-I or -II) for 24 h before co-transfection with Gag-HaloTag and Gag-NanoLuc expression vectors. At 48 h after transfection, NanoBRET signals were measured. (**c**) HCT116 cells were treated with either siCtrl or APC-targeted siRNAs (siAPC-I or -II) for 24 h before transfection with pNL4-3. At 48 h after transfection, culture supernatants and cell lysates were analysed by p24 enzyme-linked immunosorbent assay (ELISA) and immunoblotting, respectively. (**d**,**e**) Multi-cycle viral replication analysis of Jurkat cells (**d**) or primary $CD4^+$ T cells (**e**) stably expressing control shRNA (shCtrl) or APC-targeted shRNA (shAPC). Cells were infected with HIV-1_{NL4-3}. Cell supernatants were collected at the indicated time points and subjected to p24 ELISA. The expression of APC and Gag in each cell is also shown. All graphs are presented as a mean ± s.d. ($n = 3$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, two-tailed unpaired *t*-test. Full images for all immunoblots are provided in Supplementary Fig. 5.

However, when co-expressed with pNL4-3-pol-MS2x24, these GFP signals were also found in the cytoplasm and at the PM (Supplementary Fig. 2b), indicating that the vRNA had been labelled by MS2–GFP. We analysed for the subcellular localization of vRNA over 20 cells in the presence or absence of APC. By using this system, we could detect a certain level of vRNA at the PM in control cells. Notably, this localization was significantly abolished in APC-depleted cells (Fig. 6d,e; Supplementary Fig. 2c), while the localization of non-viral RNA (luciferase RNA fused with MS2x24 (ref. 37)) was not affected by the APC depletion (Supplementary Fig. 3). These results demonstrated that APC regulates PM localization of vRNA, resulting in the efficient packaging of vRNA into virions for sustained viral infectivity.

APC regulates cell-to-cell viral transfer. We finally assessed if APC is involved in the topology and spread of viral progeny in polarized cells. To this purpose, we investigated whether APC regulates the VS-mediated cell-to-cell viral transfer. In HIV-producing T cells, we found that accumulated Gag, vRNA and also APC at cell-to-cell contact sites that could be highlighted by a VS marker GM1 (ref. 38; Fig. 7a–c). Notably, APC depletion

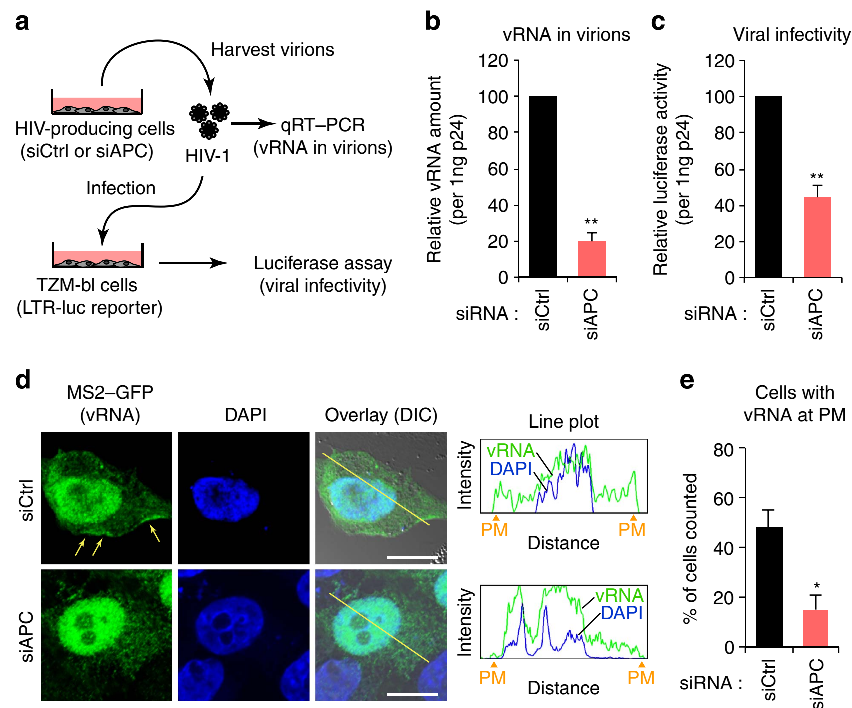


Figure 6 | APC regulates the PM localization and viral packaging of HIV-1 vRNA. (**a**) Schematic representation of virions from HIV-producing cells. HEK293 cells were treated with either control siRNA (siCtrl) or APC-targeted siRNA mix (siAPC) for 24 h before transfection with pNL4-3. After 48 h, virus-containing supernatants were subjected to RNA extraction or used to infect TZM-bl reporter cells to measure vRNA levels or viral infectivity, respectively. (**b**,**c**) APC depletion reduces the level of vRNA in virions (**b**) and also suppresses viral infectivity (**c**). Data were normalized to p24 antigen levels in the supernatants. (**d**,**e**) HeLa cells were treated with either siCtrl or siAPC for 24 h before co-transfection with pNL4-3-pol-MS2x24 and MS2–GFP expression plasmids. At 48 h after transfection, cells were stained with anti-GFP and 4,6-diamidino-2-phenylindole (DAPI) (**d**, left). Scale bar, 10 μm. The arrows indicate the localization of vRNA at the plasma membrane (PM). Line plots in the right panels in **d** indicate the fluorescence intensity with regions of the PM. The bar chart indicates the percentage of cells (at least 20 cells selected from random fields) that vRNA was found at the PM. All graphs are presented as a mean ± s.d. ($n=3$). *$P<0.05$, **$P<0.01$, two-tailed unpaired t-test.

significantly reduced the localization of these viral components at cell-to-cell contact sites (Fig. 7a–c). To monitor cell-to-cell HIV-1 transmission, we used a mixed cell culture model. As virus-producing cells, Jurkat T cells were initially transduced with control or APC-targeted shRNA, and then transfected with pNL4-3/Gag–enhanced green fluorescent protein (EGFP), in which GFP was fused to the C terminus of Gag. Uninfected target Jurkat T cells were transiently labelled with Calcein Red-Orange fluorescein dye and then co-cultured with the virus-producing cells in U-bottom plates for 24 h, GFP signals in target cells were detected by flow cytometry to measure cell-to-cell virus transfer (Fig. 7d,e). Using this system, we could quantify the GFP signals that had transferred from producer cells to target cells via VS[39]. We found that the HIV-1 transmission efficiency was prominently reduced when APC was depleted in producer cells (Fig. 7f). Moreover, we performed a cell-to-cell virus transfer experiment using HIV-infected primary CD4$^+$ T cells. We utilized LuSIV cells[40] as target reporter cells for the detection and quantitation of HIV-1 transfer due to the relatively low virus replication efficiency in primary CD4$^+$ T cells (Fig. 7g). Our results demonstrated that targeted depletion of APC in HIV-infected primary CD4$^+$ T cells prominently reduces the virus transfer (Fig. 7h). Taken together, our data suggest that APC regulates cell-to-cell viral transfer in CD4$^+$ T cells by enhancing the targeting of Gag and vRNA to the VS.

Discussion

Retroviruses are fully reliant on the host machinery for progeny production and efficient transmission. Accumulating evidence suggests that the spatial organization of host cells can create a guidance system for dynamic and intensive virion spread[41]. In our current study, we identified the tumour suppressor protein APC as a crucial host factor that orchestrates the assembly and infectious particle production required for directional HIV-1 spread. APC was found to bind and functionally stabilize both Gag and vRNA at specific PM structures such as the VS, resulting in the efficient HIV-1 particle production and spread. Our current study thus unveils a novel role of APC in the topological assembly of HIV-1 that is synchronized with the host cellular architecture and function.

There are two main modes of HIV-1 infection and propagation, both of which are dependent on the host system. Apart from cell-free infection by released viral particles, HIV-1 can spread when nascent viruses are passed directly from an infected cell to a neighbouring uninfected cell. This cell-to-cell infection that arises via the intercellular spatial structure known as a VS is a mode of HIV-1 spread that is several thousand-fold more efficient than cell-free infection[18]. This is therefore a major mode of infection in lymphoid tissues[17,42]. Cell-to-cell infection is likely relevant to the establishment of a viral reservoir[43] and also to the death of CD4$^+$ T cells induced by HIV-1 (ref. 44). The formation of a VS is required for the interaction between HIV-1 Env (gp120) on infected cells and CD4 on target cells, a process that is accompanied by the rearrangement of actin filaments leading to dynamic changes in cell polarity[17,45]. Hence, the VS-oriented accumulation of viral and cellular components in HIV-1-producing cells should be necessary for efficient cell-to-cell infection. Our current study thus uncovers a distinct function of APC in regulating the directional assembly/budding of HIV-1

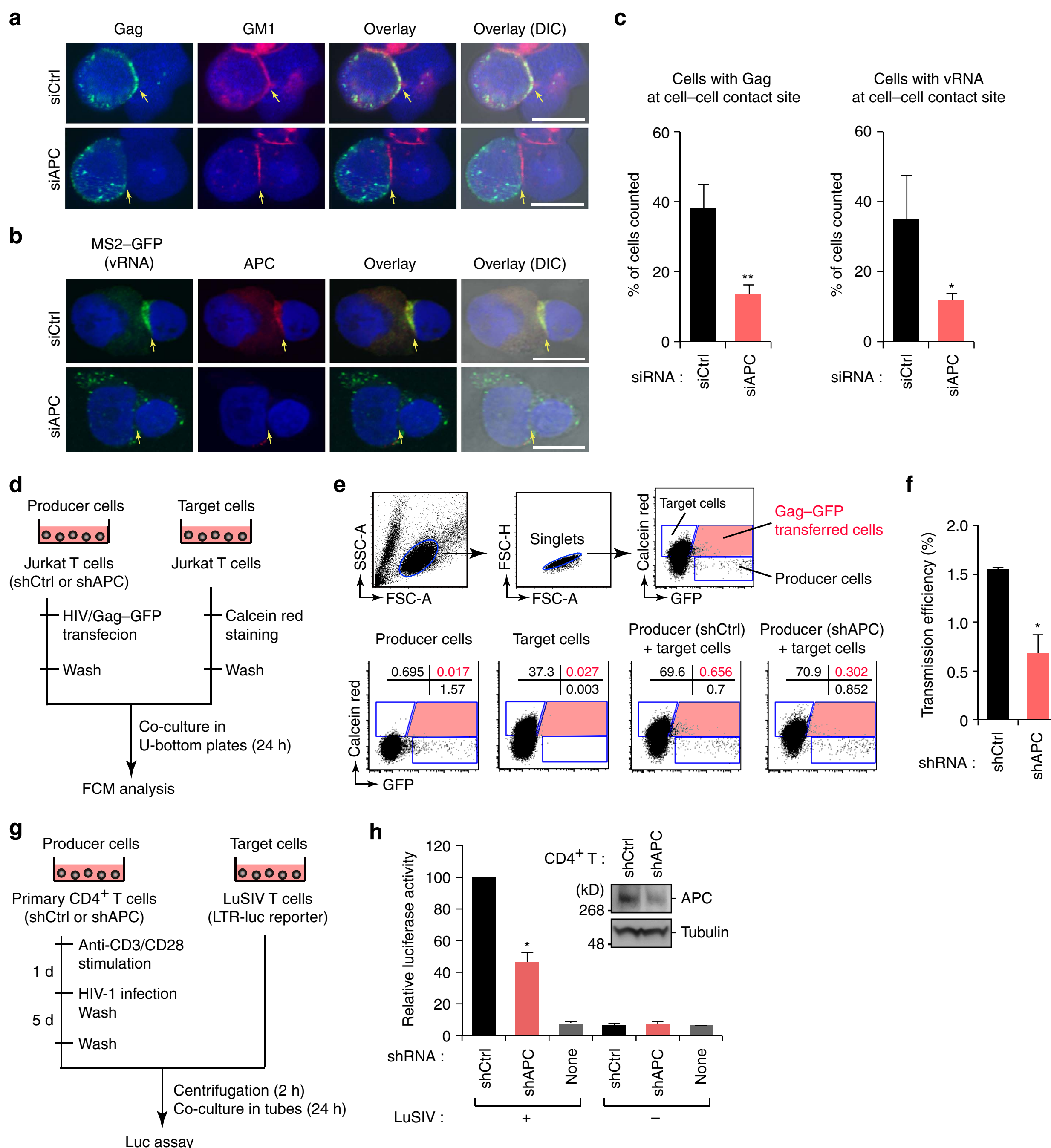


Figure 7 | APC regulates cell-to-cell viral transfer via virological synapses. (**a**,**c**) Confocal microscopic analysis of A3.01 cells expressing pNL4-3/Gag-EGFP and either control siRNA (siCtrl) or an APC-specific siRNA mix (siAPC). Cells were stained with Alexa594-conjugated Cholera Toxin Subunit B targeting cell surface GM1 ganglioside (red) and 4,6-diamidino-2-phenylindole (nuclei, blue). Scale bar, 10 µm. The arrows indicate the cell-cell contact site. The bar chart (**c**, left) indicates the percentage of cells (at least 50 cells selected from random fields) that Gag was found at the cell-cell contact sites. (**b**,**c**) Confocal microscopic analysis of A3.01 cells co-expressing pNL4-3-pol-MS2x24, MS2-GFP and either siCtrl or siAPC. Cells were stained with anti-GFP (vRNA, green) and anti-APC (red). Scale bar, 10 µm. The arrows indicate the cell–cell contact site. The bar chart (**c**, right) indicates the percentage of cells (at least 50 cells selected from random fields) that vRNA was found at the cell–cell contact sites. (**d**–**f**) Schematic representation of FACS-based cell-to-cell viral transfer assays used in this study. Jurkat cells stably expressing control shRNA (shCtrl) or APC-targeted shRNA (shAPC) were transfected with pNL4-3/Gag–EGFP, and were used as viral producer cells. Uninfected Jurkat cells stained with Calcein Red-Orange fluorescent dye were used as target cells. Two cell lines were co-cultured in a U-bottom plate for 24 h, and fluorescence signals were then measured by flow cytometry. The transmission efficiency shown in **f** was calculated from the percentage of GFP$^+$ cells among the Calcein-labelled cells by flow cytometry. Data were normalized with the number of virus producer cells. (**g**,**h**) Schematic representation of luciferase-based cell-to-cell viral spread assays used in this study. Primary CD4$^+$ T cells stably expressing shCtrl or shAPC were infected with HIV-1$_{NL4-3}$ (50 ng of p24 antigen) for 5 days, and were used as viral producer cells. LuSIV cells, a reporter cell line for HIV infection with Tat-mediated expression of luciferase, were used as target cells. These cells were co-cultured for 24 h and subjected to luciferase activity (**h**). The expression of APC in cells is also shown. Full images for all immunoblots are provided in Supplementary Fig. 5. All graphs are presented as a mean ± s.d. ($n = 3$). *$P < 0.05$, **$P < 0.01$, two-tailed unpaired t-test.

and resultant cell-to-cell viral transmission, that could provide a new mode of therapeutic intervention against HIV-1 infection that targets virus–host interactions.

Another function of APC that has been reported previously is a linking of the intracellular transport and subsequent translation of specific messenger RNAs in intracellular clusters of polarized cells, such as the presynaptic axons of neurons[46]. In retroviral infection, the accumulation of vRNA at the site of assembly is thought to be crucial for its packaging into virions. However, it has remained largely unknown how vRNA is concentrated at virus assembly sites during the late stage of the viral life cycle of HIV-1. In our current study, we have demonstrated a crucial role of APC in the vRNA localization pathway. We found that APC regulates the localization of vRNA at the cell periphery like in VSs where Gag is assembled. Furthermore, we found that APC facilitates vRNA packaging into the virions released in the culture supernatant. We thus hypothesize from our new findings that APC may regulate the localization and functional stabilization of Gag at the VS thereby enhancing its binding to vRNA for its incorporation into HIV-1 virions. Indeed, it has been reported that vRNA can stably localize at the PM in the presence of Gag[12,13]. Another study has also reported that even in the absence of Gag, vRNA can still reach the PM via a diffusion mechanism, but its movement in this case is unstable[47]. We cannot yet completely exclude the possibility that APC directly binds vRNA to enhance its intracellular trafficking, membrane localization and packaging into virions. Further careful analysis will be required to more precisely determine the molecular function of APC with regard to vRNA during HIV-1 particle production.

Gag MA contains a HBR composed mainly of basic amino acids as a bipartite membrane-binding motif[7,8,48,49]. Earlier studies have demonstrated that mutation of HBR causing mislocalization of Gag to intracellular compartments resulted in lower levels of HIV-1 production[48–51]. The binding of the HBR to PI(4,5)P_2 has been shown to contribute to PM targeting of the Gag polyprotein[28]. However, PI(4,5)P_2 is highly enriched not only at the PM, but also at the rim of caveolae and in coated pits[52]. Recently, host transfer RNA has been found to bind to Gag MA in a manner dependent on the electrostatics of the HBR, while this interaction seems to interfere the PM targeting of Gag[11,53]. Hence, there may be another mechanism of HBR-mediated Gag stabilization at the PM. Otherwise Gag will be missorted to endosomal compartments even after its PM targeting. Our current findings demonstrating that APC prefers to interact with the Gag MA, but not its HBR mutant with neutral amino acids, provide a new concept that the basic amino acids in HBR are relevant to Gag stabilization at the PM, mediated by the function of APC.

The *APC* gene at chromosome 5q21 is transcribed into a nearly 9.5 kb-sized messenger RNA, whose mutations cause FAP and colorectal cancer[54]. The mutation cluster region is located at the central part of the *APC* gene, and the high rates of somatic mutations in this region lead to a premature stop codon, resulting in a nonfunctional truncated APC protein lacking its C-terminal half. In fact, ~80% of FAP patients have truncating mutations in the mutation cluster region of the *APC* gene[55]. APC has a well-recognized function in regulating the Wnt/β-catenin signalling pathway, but its truncated mutants found in FAP patients have completely lost this ability in a dominant-negative manner[56]. Our current analyses suggest that a FAP-related APC mutant does not support HIV-1 production. Conversely, expression of the C-terminal end of APC exhibits a dominant-negative effect upon HIV-1 production, suggesting that the N-terminal and/or middle regions of APC is a prerequisite for its functional regulation of HIV-1. Since human genetic variation is known to affect host susceptibility to HIV-1 infection and disease progression following infection[57], more comprehensive genetic epidemiological studies will be necessary to further explore the biological consequences of APC mutations in HIV-1 infection.

APC plays a central role in suppressing the canonical Wnt signalling pathway that controls cell proliferation and differentiation not only in the intestine[58] but also in the immune system[59]. Previous studies have shown that Wnt signalling is a crucial pathway that regulates many aspects of T-cell biology, including their differentiation and development into effector T cells. In addition to the native function of APC in regulating T cells, we have demonstrated a distinct role of APC in the directional assembly and spread of HIV-1. Interestingly, our current data indicates that Wnt/β-catenin signalling does not directly contribute to the role of APC in HIV-1 production. This unexpected finding evokes the principle that HIV-1 hijacks the host cellular machinery to reproduce itself. However, the dependency of HIV-1 on APC in immune cells could be a promising target for the development of antiviral therapies in the future.

Methods

Plasmids. Human APC genes (UniProt ID #P25054) were amplified from previously described plasmid (pCMV-APC)[60] and subcloned into the pEGFP vector (Clontech, Palo Alto, CA). The mApple-tagged APC was kindly contributed by Dr. Yuko Mimori-Kiyosue (RIKEN, Kobe, Japan). The human-codon-optimized HIV-1 Gag complementary DNA (cDNA) were amplified from previously described plasmid (pGEX-2T-Gag)[61] and subcloned into the pcDNA3 (Thermo Fisher Scientific, Waltham, MA), pCI, pHTC, pNLF1-C (Promega, Madison, WI) and pEGFP (Clontech) vectors. Detailed information regarding the Gag fusion constructs and HIV-1 molecular clones used in the analysis is provided in Supplementary Table 2. For AlphaScreen analysis, APC and Gag cDNAs conjugated with C terminally FLAG and GST-bls (biotin-ligating sequence) tag were subcloned into the pEU-based vector (CellFree Sciences, Ehime, Japan). To visualize vRNA, 24 repeats of the MS2-binding sequence (24xMBS; ACATGAGGATCACCCATGT) were inserted at the EcoRV/AgeI restriction site in the *pol* gene of pNL4-3 carrying the D25N mutation in the protease region. As a non-viral control, a 24xMBS cassette was inserted at the XbaI restriction site at downstream of the stop codon of luciferase gene of pGL4.73 vector (Promega). The expression plasmids for GFP fused to MS2 were obtained from Addgene (#27121).

Cells and viruses. HEK293 (#CRL-1573), HeLa (#CCL-2), HCT116 (#CCL-247), A549 (#CCL-185, obtained from American Type Culture Collection; ATCC) and TZM-bl (#8129, from NIH AIDS Reagent Program; ARP) cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS). SW480 (#CCL-228), Jurkat (#TIB-152, from ATCC) and A3.01 (#166, from ARP) cells were cultured in RPMI containing 10% FBS. ACH-2 (#349) and LuSIV (#5460, from ARP) cells were cultured in RPMI containing 10% FBS, 2 mM sodium pyruvate and 10 mM HEPES. Primary human $CD4^+$ T cells (#2W-200, purchased from Lonza, Basel, Switzerland) were maintained in LGM-3 medium (Lonza) supplemented with 10% FBS. These cells were confirmed to be free of mycoplasma using a MycoAlert mycoplasma detection kit (Lonza). Replication-competent HIV-1 stocks were produced by transient transfection of HEK293 cells with the pNL4-3 proviral plasmid (#114, from ARP)[25]. Culture supernatants containing virus were collected 48 h after transfection and filtered through a 0.45 μm Millex-HV filter (Millipore, Billerica, MA).

RNA interference and antibodies. In siRNA experiments, cells were transfected with APC-targeted siRNAs (#HSS100547 and #HSS179353) or control siRNA (#12935300, both from Thermo Fisher Scientific) using Lipofectamine RNAiMAX (Thermo Fisher Scientific) or Hiperfectamine transfection reagent (Qiagen, Venlo, Netherlands) 1 day before DNA transfection or virus infection. Stably APC-silenced cells were generated by transduction with lentiviral particles carrying APC-targeted shRNA (#sc-29703-V) or control shRNA (#sc-108080, both from Santa Cruz Biotechnology, Dallas, TX), and then were selected with 1 $\mu g\,ml^{-1}$ (Jurkat cells) and 2.5 $\mu g\,ml^{-1}$ (primary $CD4^+$ T cells) puromycin for 7–10 days. Primary $CD4^+$ T cells were stimulated with anti-CD3/CD28 beads (Dynabeads Human T-Activator, Thermo Fisher Scientific) in the presence of 30 $U\,ml^{-1}$ interleukin-2 (Miltenyi Biotec, Bergisch Gladbach, Germany) 1 day before the transduction of the lentiviral particles. The antibodies used in this study are listed in Supplementary Table 3.

Proteomic analyses. HEK293 cells expressing Gag C terminally fused to TAP tag were lysed with IPP150 buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1%

NP-40) supplemented with 10 mM Na_3VO_4, 10 mM NaF and protease inhibitor Complete mini (Roche Diagnostics, Basel, Switzerland). Cell lysates were incubated with IgG sepharose beads (GE Healthcare, Little Chalfont, UK) for 2 h at 4 °C. Beads were washed and incubated with TEV protease (Thermo Fisher Scientific) in TEV cleavage buffer (IPP150 containing 0.5 mM EDTA and 1 mM DTT) for 16 h at 4 °C. The TEV cleavage products was then incubated with calmodulin beads in calmodulin-binding buffer (IPP50 containing 10 mM 2-mercaptoethanol, 1 mM magnesium acetate, 1 mM imidazole and 2 mM calcium chloride) for 1 h at 4 °C. Proteins were eluted with TAP elution buffer (IPP150 containing 10 mM 2-mercaptoethanol, 1 mM magnesium acetate, 1 mM imidazole and 2 mM EGTA) and separated by SDS–PAGE and visualized by silver staining. For in-gel digestion, gel bands were excised from the silver-stained gel, incubated with 50 mM ammonium bicarbonate/60% acetonitrile for 30 min, and then dehydrated. Gel slices were incubated with 12.5 ng μl^{-1} trypsin (Promega) in 50 mM ammonium bicarbonate for 16 h at 37 °C. Peptides were eluted from the gel slices with 0.2% formic acid, and then filtrated through a 0.22 µm Ultrafree-MC (Millipore). Mass spectrometric analysis was performed on a LTQ Orbitrap Velos hybrid mass spectrometer (Thermo Fisher Scientific). Protein identification was performed using the Mascot program (Matrix Science, London, UK).

***In vitro* protein binding assays and AlphaScreen analysis.** In the GST pull-down assays, GST-tagged Gag was expressed in *Escherichia coli* BL21 (DE3) cells (#2527I, New England Biolabs, Ipswich, MA) and purified using standard protocols. HEK293 cell lysates were incubated with glutathione beads that had been coupled with Gag–GST proteins. The beads were then washed, and bound proteins were subjected to immunoblotting analysis, as previously described[34,62]. Immunoprecipitation analysis was also performed as previously described[63]. Briefly, cell lysates were immunoprecipitated with 2 µg of antibodies mixed with protein G sepharose (GE Healthcare). In the experiment using ACH-2 cells (HIV-1 latent T-cell clone), cells were stimulated with phorbol 12-myristate 13-acetate (PMA) (100 ng ml^{-1}) to reactivate HIV-1 gene expression 2 days before immunoprecipitation. Wheat germ cell-free protein production and AlphaScreen analysis was also performed as described previously[64,65]. Briefly, DNA templates containing FLAG or GST-bls epitope were amplified by PCR with pEU-based vectors and corresponding primers, and then were subjected to the protein production process using the protein synthesizer DTII (CellFree Sciences). AlphaScreen signals from the mixture were detected with an EnVision device (PerkinElmer, Waltham, MA).

Transfection-based HIV-1 production assays. Cells in 12-well plates were co-transfected with the HIV molecular clone (100 ng) and either an APC expression vector or empty vector (250 or 500 ng). In the siRNA experiments, cells were transfected with siRNA (20 pmol) 1 day before the transfection of the HIV molecular clone. Two days after transfection, cell lysates and supernatants were collected and subjected to immunoblotting analysis. The p24 antigens in the supernatants were measured with an ELISA kit (Zepto Metrix, Buffalo, NY). To calculate viral infectivity, TZM-bl reporter cells were infected with normalized viruses (1 or 5 ng of p24 antigen) and the HIV-1 long terminal repeat (LTR)-driven luciferase activity was assayed at 2 days post infection. For vRNA detection, total RNA was extracted with TRIzol (Thermo Fisher Scientific) and further treated with DNase I (Takara Bio, Shiga, Japan). After phenol:chloroform extraction and subsequent ethanol precipitation, cDNA was generated with ReverTra Ace (Toyobo, Osaka, Japan) and an oligo$(dT)_{20}$ primer, and quantitative PCR was then performed using SYBR premix Taq (Takara Bio). The PCR primers were as follows: 5′-CATGTTTTCAGCATTATCAGAAGGA-3′ (vRNA-Gag6-Fwd) and 5′-TGCTT GATGTCCCCCCACT-3′ (vRNA-Gag84-Rev)[66].

Multi-cycle HIV-1 replication assays. Jurkat (10^5 cells) or primary CD4$^+$ T cells (10^6 cells) stably expressing either shCtrl or shAPC were infected with HIV-1_{NL4-3} (25 ng of p24 antigen). The latter cells were stimulated with anti-CD3/CD28 beads in the presence of 30 U ml^{-1} interleukin-2 1 day before HIV-1 infection, as described above. The infection was performed by centrifugation for 90 min at 500 g in the presence of polybrene (5 µg ml^{-1} •), and cells were then washed to remove the input viruses. Nascent virions produced from infected cells were collected periodically and the p24 levels were measured as described above.

Microscopic analysis. For immunofluorescence, HeLa or A549 cells were seeded onto glass cover slips 1 day before transfection. At 24 or 48 h post transfection, the cells were fixed with 4% paraformaldehyde (PFA) and permeabilized with 0.5% Triton X-100. The cells were then stained with primary antibodies and Alexa Fluor-conjugated secondary antibodies. In the experiments using A3.01 T cells, transfected cells were cultured in round-bottom plates for 48 h. Cells were then fixed with 4% PFA and permeabilized with 0.2% saponin (except for surface GM1 staining), and stained with antibodies. For GM1 staining, fixed cells were stained with Alexafluor594-conjugated Cholera Toxin Subunit B (Thermo Fisher Scientific) for 30 min. Microscopic imaging was performed with an FV1000-D confocal laser scanning microscope (Olympus, Tokyo, Japan). Line plots of the fluorescence intensity were generated by ImageJ software (NIH, Bethesda, MD).

Quantitative analysis of subcellular localization of Gag–GFP was performed as previously reported[34,67,68]. Briefly, HeLa cells expressing Gag–GFP were fixed and 20 random fields were inspected. Over 100 cells were analysed for the subcellular localization of Gag–GFP, which was either strongly evident at the PM only, at the PM with intracellular accumulations, or diffusely in the cytoplasm.

Cell-to-cell viral transfer assays. For fluorescence-activated cell sorting-based analysis, Jurkat cells stably expressing either shCtrl or shAPC (10^5 cells) were transfected with 5 µg of pNL4-3/Gag–EGFP[67] using the Neon transfection system (Thermo Fisher Scientific) in accordance with the manufacturer's protocol. Uninfected Jurkat cells (10^6 cells) were labelled with 100 nM of fluorescein dye (CellTrace Calcein Red-Orange AM, Thermo Fisher Scientific) for 20 min at 37 °C, and were used as target cells. These cells were mixed and centrifuged at 500 g for 2 h, and then cultured in U-bottom 96-well plates for 24 h. Cells were fixed with 4% formaldehyde and analysed using a FACSCanto II instrument (BD Biosciences, San Jose, CA). Data were analysed with FlowJo software (Treestar, Ashland, OR). We excluded cell–cell doublets by detecting disproportions between forward scatter (FSC)-A versus FSC-H to analyse the exact efficiency of viral transfer. The transmission efficiency was calculated from the percentage of GFP$^+$ cells among the Calcein-labelled cells. Data were normalized with the number of virus producer cells.

For luciferase-based analysis, primary CD4$^+$ T cells stably expressing either shCtrl or shAPC (10^5 cells) were infected with HIV-1_{NL4-3} (50 ng of p24 antigen) for 2 h. Five days later, these cells were mixed with a same number of LuSIV cells (a human T-cell line containing LTR-driven luciferase gene) and then centrifuged at 500 g for 2 h. After co-culturing in a 1.5- ml tube for 24 h at 37 °C, the cells were assayed for luciferase activity.

NanoBRET assays. Cells in 96-well plates were transfected with Gag-NanoLuc and Gag-HaloTag expression vectors at a ratio of 1:100. At 48 h post transfection, NanoBRET activity was measured using the NanoBRET Nano-Glo Detection System (Promega). If both proteins are within 100 Å, NanoBRET signals are detected[36].

Statistical analysis. The values in all graphs are presented as a mean and s.d. The statistical significance of differences between two groups was tested using a two-tailed unpaired *t*-test with Prism 6 software (GraphPad, San Diego, CA). A P value of <0.05 was considered statistically significant.

Data availability. All relevant data are available from the authors on request.

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Author contributions
K.M. designed and performed the research, analysed the data and wrote the manuscript; M.N., S.M., A. Okayama, M.A. and A.K. performed the research and analysed the data; H.H., Y.M., and A. Ono contributed reagents and analysed the data; H.K. and N.Y. designed the research and analysed the data; and A.R. designed and supervised the research, analysed the data, and wrote the manuscript.

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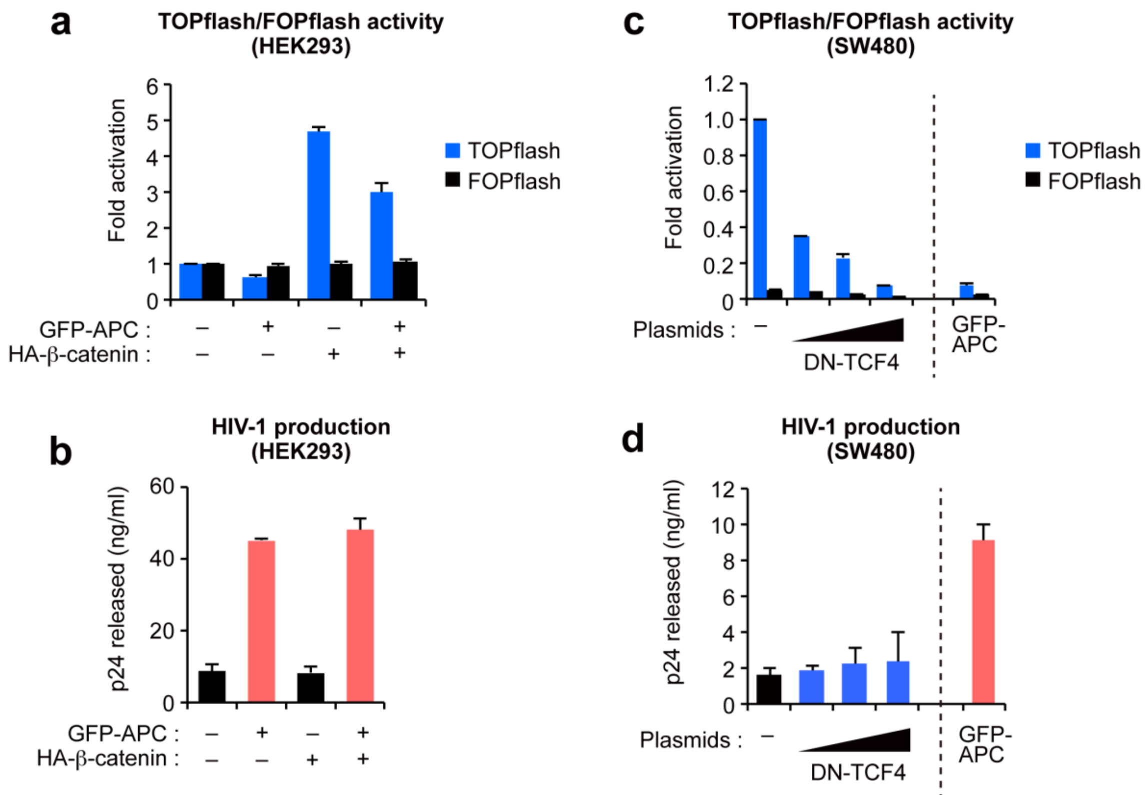
Supplementary Figure 1

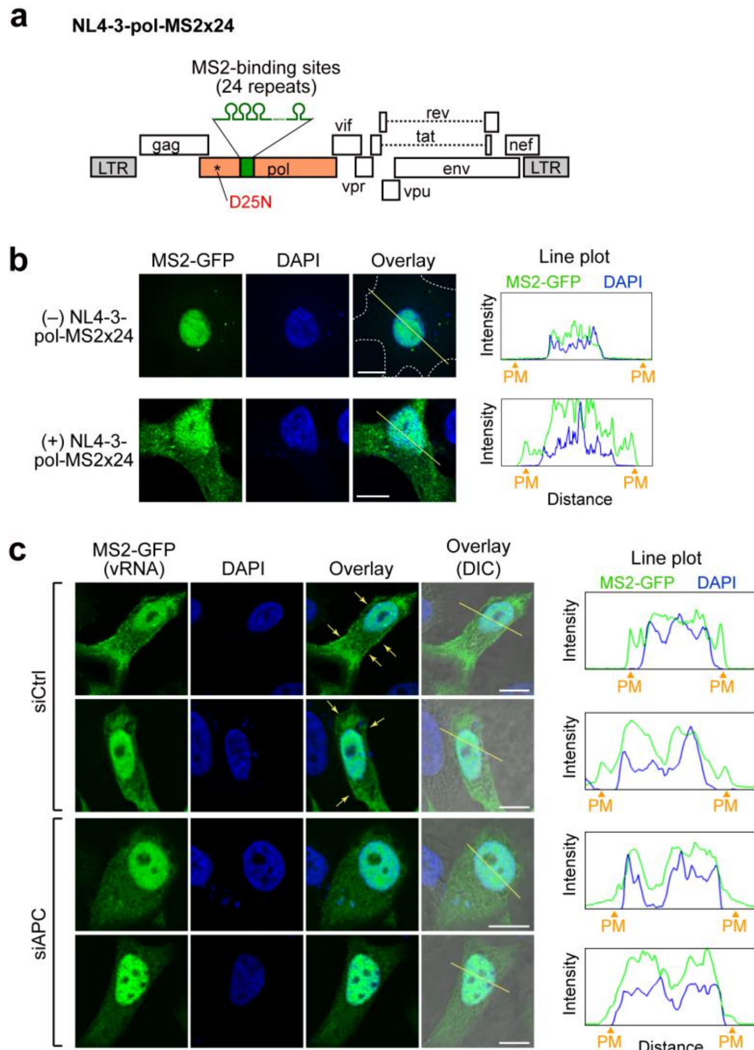


Supplementary Figure 1. Wnt/β-catenin signaling does not affect APC function in HIV-1 production.

(a, b) TOPflash (TCF4-luc) reporter assay (a) and HIV-1 production assay (b) of HEK293 cells expressing β-catenin and APC. FOPflash vector (TCF4-mut-luc) was also used as a negative control. HEK293 cells were co-transfected with pNL4-3 and GFP-APC in the presence or absence HA-tagged β-catenin. The bar chart in (b) indicates the viral p24 antigen levels in the culture supernatants. Note that the expression of β-catenin had no observable effects on APC-mediated HIV-1 production while it significantly increased TCF4-dependent transcriptional activity.

(c, d) SW480 cells were transfected with either TOPflash or FOPflash and co-transfected with different amounts of dominant-negative (dn)TCF4 or GFP-APC, followed by a gene reporter assay (c). SW480 cells were co-transfected with pNL4-3 together with different amounts of dnTCF4 or GFP-tagged APC. The bar chart in (d) indicates the viral p24 antigen levels in the culture supernatants. Note that the expression of dnTCF4 in SW480 cells (APC-mutated colorectal cancer cells) had no observable effects on the HIV-1 production, whereas it significantly reduced TCF4-dependent transcriptional activity. All graphs are presented as a mean ± s.d. (n = 3).

Supplementary Figure 2



Supplementary Figure 2. vRNA labeling system used in this study.

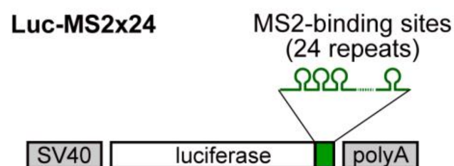
(a) Schematic representation of the HIV-1 gene tagged with twenty-four repeats of MS2-binding sites (MBS) used in this study. MBS were inserted into a Pol gene with no protease activity due to a D25N mutation in the protease region.

(b) HeLa cells were co-transfected with the MS2-GFP expression plasmid with or without pNL4-3-pol-MS2x24. Cells were stained with anti-GFP antibody to highlight MS2-GFP signals, and the nuclei were counterstained with DAPI (blue). The dotted line denotes the cell border. Scale bar, 10 μm. Line plots in the right panels indicate the fluorescence intensity of MS2-GFP (green) and DAPI (blue) within regions of the plasma membrane (PM).

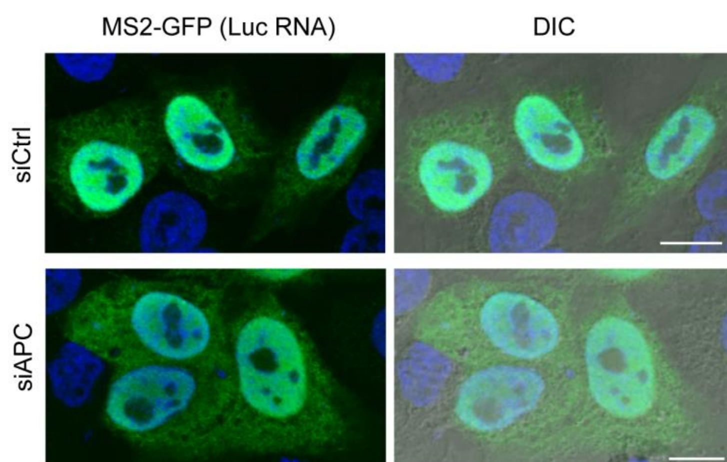
(c) Other representative example of cell images shown in Figure 6d.

Supplementary Figure 3

a

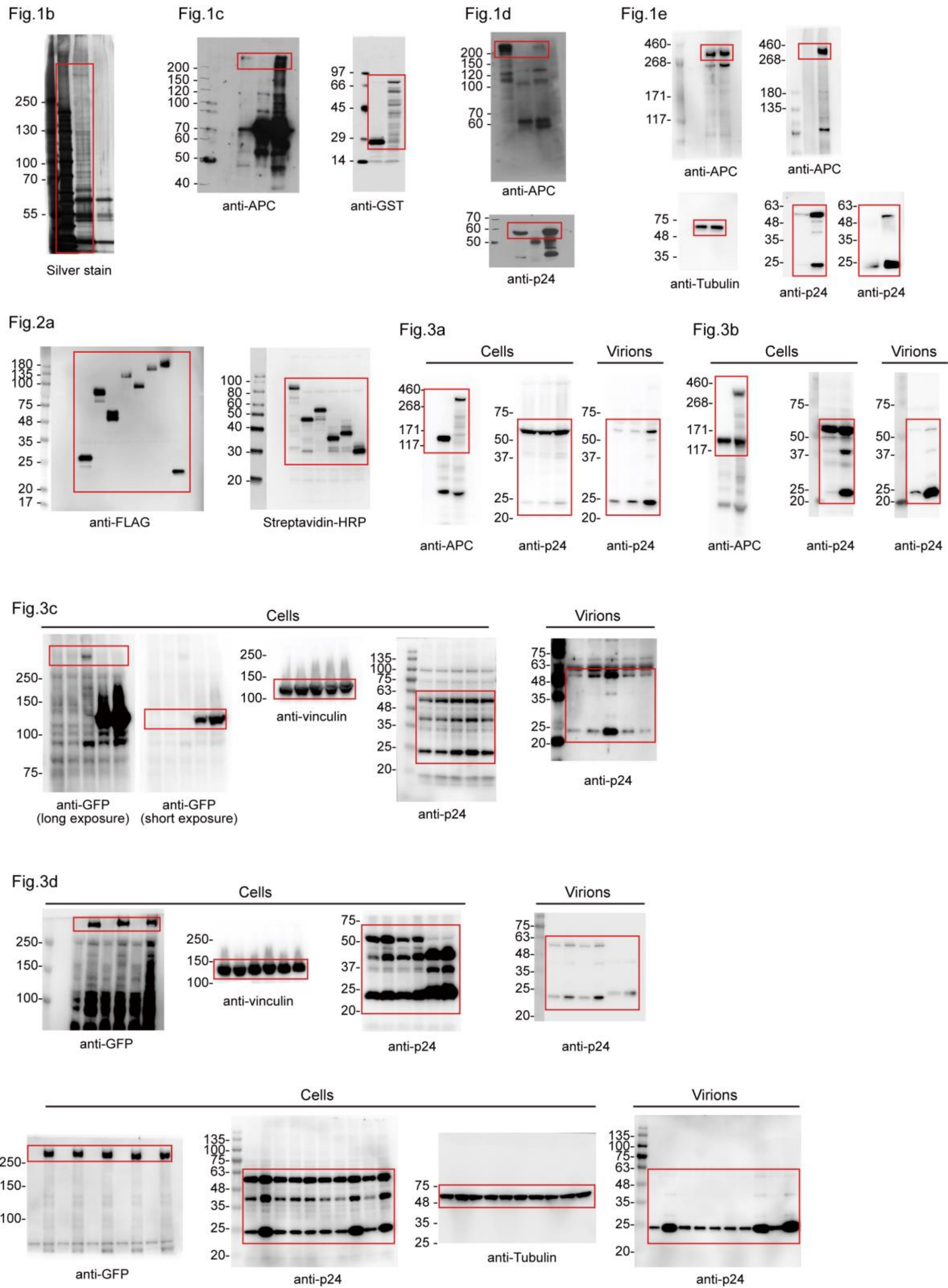


b



Supplementary Figure 3. APC does not affect non-retroviral RNA.
(a) Schematic representation of a non-viral (Renilla luciferase) RNA construct tagged with twenty-four repeats of MS2-binding sites (MBS). MBS were inserted at the end of luciferase gene of pGL4.73 (SV40-driven luciferase expression vector, obtained from Promega).
(b) HeLa cells were treated with control siRNA (siCtrl) or APC-targeted siRNA mix (siAPC) for 24 h prior to co-transfection with a pLuc-MS2x24 and MS2-GFP expression plasmid. Cells were stained with anti-GFP antibody and with DAPI (blue). Scale bar, 10 μm.

Supplementary Figure 4



Supplementary Figure 4. Full images of the immunoblots presented in Figures 1-3.

Supplementary Figure 5

Fig.4a

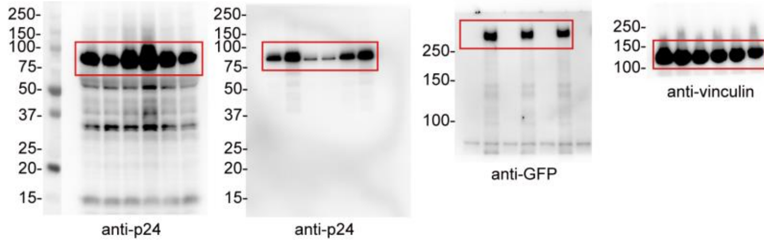


Fig.5a

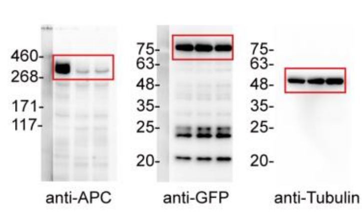


Fig.5c

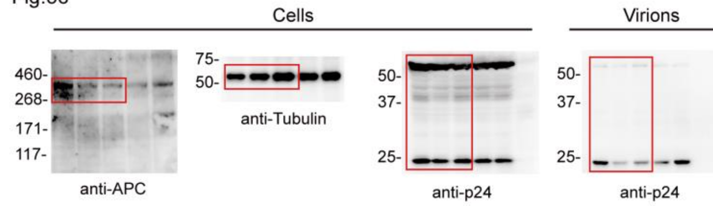


Fig.5d,e

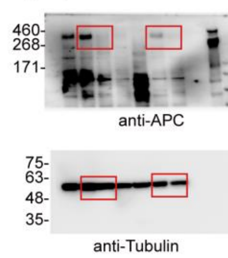


Fig.5d

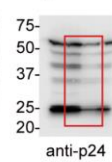


Fig.5e

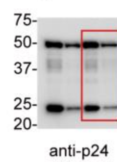
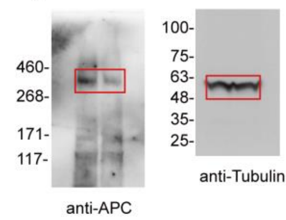


Fig.7f



Supplementary Figure 5. Full images of the immunoblots presented in Figures 4-7.

Supplementary Table 1

Gel slice [a]	Gene symbol	M.W. [b]	Protein score [c]	Protein matches [d]	Peptide matches [e]	Previously reported HIV interactions [f]	Reference
#1	**APC**	**311.6**	**20**	**7**	**1**	–	
	LAMA3	366.7	17	6	2	Env, Tat	
#2	**NUMA1**	**238.1**	**614**	**44**	**18**	Gag-Pol, Tat	1
	MYH10	228.9	76	16	2	Gag, Tat	2
	BAZ2A	211.1	44	13	3	Vif	
	SEC16A	233.5	33	11	1	–	
	PARP12	226.3	19	4	1	–	
#3	**CLTC**	**191.5**	**674**	**32**	**24**	Gag, Pol, Env, Vpr	3
	CHD1	196.6	66	15	4	–	
	NUP188	196.0	62	7	3	–	
	ARMS	196.4	41	6	2	Tat	
	DNMT1	183.1	38	12	3	Tat	
	TAB182	181.7	26	16	3	–	
#4	**AMOT**	**118.0**	**1039**	**66**	**41**	Gag	4
	UBAP2L	114.5	313	10	9	Gag	2
	NAT10	115.7	275	21	12	Gag, Rev	2, 5
	DHX36	114.7	88	5	3	Rev	
	HLTF	113.9	81	9	4	Nef	
	CNTN1	113.3	49	5	2	–	
	DDX46	117.4	24	8	1	Tat	
	PSD3	116.0	23	8	1	–	
	RNF20	116.0	21	5	2	Gag, Env, Nef	3
#5	**HSP90B1**	**92.4**	**62**	**4**	**2**	–	
	MOGS	91.9	39	4	2	Gag, Pol, Env, Vpr, Nef	6
	KANK2	91.2	29	2	1	–	
	MCM6	92.8	25	4	1	–	
	AMPD3	88.8	16	7	1	–	
	ZNF616	90.3	21	10	1	–	

[a]Gel slice numbers indicated in Figure 1b. [b]Molecular weight (kD).
[c]MASCOT protein score. [d]Number of assigned spectra.
[e]Total numbers of peptides sequenced.
[f]Data collected from the HIV-1 Human Interaction Database (www.ncbi.nlm.nih.gov/genome/viruses/retroviruses/hiv-1/interactions/).

Supplementary Table 1. Gag-associated proteins identified by mass spectrometry.

Supplementary Table 2

Plasmids	Gene/Insert	References
pcDNA3-Gag-TAP	Gag(huOpt*)-TAP	See below
pCI-Gag-FLAG	Gag(huOpt)-FLAG	See below
pEGFP-N-Gag	Gag(huOpt)-FLAG-GFP	See below
pGEX-Gag	Gag(huOpt)-GST	7
pHTC-Gag	Gag(huOpt)-FLAG-HaloTag	See below
pNLF1-C-Gag	Gag(huOpt)-FLAG-NanoLuc	See below
pNL4-3	All HIV-1 proteins	8
pNL4-3/Fyn(10)fullMA	Fyn(10)**-Gag and the other viral proteins	9
pNL4-3/Fyn(10)ΔMA	Fyn(10)-Gag(MA-deficient) and the other viral proteins	9
pNL4-3/Fyn(10)fullMA/Gag-YFP	Fyn(10)-Gag-YFP, Tat, Rev and Nef	9, 10
pNL4-3/Fyn(10)-Gag(6A2T)-YFP	Fyn(10)-Gag(6A2T)-YFP, Tat, Rev and Nef	11
pNL4-3/Fyn(10)-Gag(RKswitch)-YFP	Fyn(10)-Gag(RKswitch)-YFP, Tat, Rev and Nef	11
pNL4-3/Gag-EGFP	Gag-EGFP (ΔPol) and the other viral proteins	12

*Human codon-optimized. **Fyn(10): N-terminal 10 amino acid derived from Fyn kinase.

Supplementary Table 2. Gag fusion constructs and HIV-1 molecular clones used in this study.

Supplementary Table 3

Antibodies/Reagents	Source (Catalog number)	Dilution
APC	Santa Cruz Biotechnology (#sc-896)	1:100 (IF)
APC	Abcam (#ab58)	1:1000 (WB)
α-Tubulin	Sigma-Aldrich (#T6199)	1:10000
Vinculin	Sigma-Aldrich (#V9264)	1:1000
HA	Roche (#11867423001)	1:1000
FLAG	Sigma-Aldrich (#F3165)	1:10000
GST	Santa Cruz Biotechnology (#sc-138)	1:10000
GFP	Wako (#012-20461)	1:100 (IF)
GFP	Roche (#11814460001)	1:1000 (WB)
HIV-1 Gag p24	NIH AIDS Reagent Program (#3537)	1:1000
Streptavidin-HRP Conjugate	GE Healthcare (#RPN1231)	1:5000
HRP-conjugated anti-mouse IgG	GE Healthcare (#NA931)	1:10000
HRP-conjugated anti-rabbit IgG	GE Healthcare (#NA934)	1:10000
Cholera Toxin Subunit B, Alexafluor594-conjugated	Thermo Fisher Scientific (#C34777)	1:200

Supplementary Table 3. Antibodies and reagents used in this study.

Supplementary References

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Reviewers' comments:

Reviewer #1 (Remarks to the Author):

Nature Communications manuscript number NCOMMS-16-15882, by Miyakawa et al. presents an intriguing analysis of the effects of the tumor suppressor APC on HIV-1 assembly and infection. The strongest aspect of the manuscript is the characterization of the MA-APC binding interaction, and the analyses of the effects of APC depletion and expression on HIV-1 virus particle release. To me, the virological synapse (VS) data are less convincing to the extent that they detract from the presentation. A revised version of the manuscript should be of interest to HIV researchers. Suggestions for revisions are as follows:
1. All constructs: Readers should not be forced to track down construct details in half a dozen papers. Please include details of exactly where all Gag fusion constructs are linked to their tags.
2. Figure 1: The authors should include a table of all Gag-interacting proteins identified by MS and their spectral count numbers, as well as what proteins were identified in control TAP tag pulldowns. This will be of interest to readers.
3. Figure 2: Did the authors use full-length APC fusions? If not, please explain. Also, based on the cartoon in panel A, it looks as though the GST tag is fused to the N-terminus of MA, which might be expected to perturb its folding. Please address this issue.
4. Figure 3: In panels a-d please show the virion immunoblots and include the Gag CA (p24), p41 and Pr55 bands on the blots. This is necessary to validate release values. Also, with the SW480 blot (panel b), the cellular Gag bands for the APC- lane are much less bright than for the APC+ lanes, which could readily explain for the lack of Gag protein release as virions. Please use a better gel. I would also note that the top of the APC- blot in panel b looks highly contrasted. Perhaps a better blot here would be in order too.
5. Figure 4: Panel A: I do not see a significant difference between the examples of diffuse and PM staining. Better examples should be used. The authors also should use less subjective measurements of diffuse, PM and PM+Int staining. Panel B: I don't find the FRET data convincing. Panel C: Please show the entire virion blot. Panel D: Did the authors score for p24 levels at later timepoints? If so, what were the results?
6. Figure 5: Assuming that "APC targets cellular mRNA to actin-rich plasma membrane protrusions" (page 9), the vRNA localization is likely to be independent of the cis-active vRNA control elements. A control 24xMS2 RNA expressed from a non-retroviral plasmid ought to be tested. It also would be useful to graph cumulative RNA (MS2-GFP) line plots (summed from 20 or so images of each type) for quantification purposes.
6. Figure 6-7: I think the data from these VS model are not so convincing, and that the figures and their discussion can be deleted.
7. Supplementary Figure 3: An analyses of Fyn(10)WT, Fyn(10)6A2T and Fyn(10)RKswitch does contribute to the manuscript, but instead of using the alphascreen, and colocalization studies, the authors should simply perform parallel studies to Figure 3a and include the results as a figure in the paper itself.
8. Discussion: Assuming Figures 6-7 are deleted, the Discussion should be re-written to reflect this.

Reviewer #2 (Remarks to the Author):

The manuscript by Miyakawa et al identifies and defines the role of the tumor suppressor APC in coordinating polarized assembly of HIV. Identified from an affinity purification/mass spectrometry approach they determine that the protein can interact with the MA domain of the Gag. Knockdown studies indicate that it facilitates particle production and recruitment of Gag to the plasma membrane. Interestingly, they also provide some data to suggest that the recruitment of viral RNA to nascent virus particles is regulated by APC. These studies are well performed and supportive of their overall hypothesis, the cell types that are best studied in this manuscript are predominantly epithelial cell lines, and at the very end, an interesting experiment with a T cell line is presented that suggested that APC can play a role in efficient cell-to-cell transfer across virological synapses formed between T cell lines. The experiments performed are generally technically sound and mostly well controlled. The manuscript would strengthened by studies to show whether APC is expressed in primary T cells, and whether knock down or knock out of APC in primary T cells has an effect on infection and cell-to-cell spread. This study implicates APC as an important new host factor that participates in the polarized localization of viral assembly to the site of cell-cell contact during virological synapse formation.

Specific Comments:

1. Would be important to evaluate the levels of endogenous APC in primary T cells and determine whether this interaction occurs within infected primary T cells.
2. Fig. 7: It is not clear whether the analysis is rigorously excluding cell-cell doublets. The study may also benefit from making stable cell lines that are expressing shRNA or have a deleted APC gene with CRISPR. It would be convincing to show that the levels of APC protein are lower in the presence of siAPC vs siCtrl.

Reviewer #3 (Remarks to the Author):

Through gain and loss of function experiments it is shown here that APC is involved in the assembly and release of HIV particles from infected cells. APC binds to the gag protein of HIV-1 and is essential for release of virions (Fig.3,4), self-association of gag proteins (Fig. 4)and localization of gag proteins and viral RNA at the plasma membrane and virological synapses (Fig. 6) as well as viral transfer to non-infected cells (Fig. 7).
As a non-virologist I consider the experimental strategy and results convincing. They establish APC as a key host-factor for HIV-1 propagation which has obvious consequences to our understanding of HIV-1 biology and will stimulate research into the molecular biology and consequences of APC action.
Points to consider:
1 It is formally possible that effects of APC gain or loss of function experiments on HIV assembly are due to APC´s role in Wnt signaling, i.e. that they are mediated by b-catenin

dependent signaling rather than or in addition to its interaction with gag. In such a scenario activation of Wnt signaling by APC knockdown or in APC mutated colorectal cancer cells would prevent HIV assembly while inhibition of wnt signaling by full-size APC would stimulate it. It should be tested whether simultaneous knockdown of beta-catenin or expression of dominant-negative TCF blocks the consequences of APC knockdown in some of the key experiments shown in Figs. 4-7. Similarly, effects of b-catenin transfection or treatment of cells with Wnt3a should be determined.
2 Please explain briefly what the pNL4-3 vector encodes for.
3 What is the purpose of the Fyn part in fig. 3d?
4. Fig. 4c and d would fit better with Figure 3
5 Fig. 6 This a collection of neat experiments. However, how can authors state that GAG, vRNA and APC are enriched in protrusion if one doesn´t see the rest of the cell? Along that line, is it possible that absence of Gag and vRNA at protrusion after APC knockdown simply reflects lack of membrane association as shown for vRNA in fig. 5?

Responses to the comments of Reviewer #1

We are grateful for the insightful and constructive suggestions by this reviewer. Reviewer#1 noted some of the intriguing effects of the tumor suppressor APC on HIV-1 assembly and infection. Furthermore, this referee also accepted the significance of the MA-APC binding interaction and the effects of APC depletion and expression on HIV-1 virus particle release. However, this reviewer thinks that the virological synapse (VS) data are less convincing to the extent that they detract from the presentation. We have responded to the concerns of Reviewer#1 below.

1. All constructs: Readers should not be forced to track down construct details in half a dozen papers. Please include details of exactly where all Gag fusion constructs are linked to their tags.

<u>Response:</u> We apologize for the lack of information regarding Gag fusion constructs. We have now added this information to our revised text (**Supplementary Table 2**).

2. Figure 1: The authors should include a table of all Gag-interacting proteins identified by MS and their spectral count numbers, as well as what proteins were identified in control TAP tag pulldowns. This will be of interest to readers.

<u>Response:</u> We apologize for the inaccurate description in our proteomic analysis. Indeed, we performed an In-Gel tryptic Digestion (IGD) of silver-stained bands excised from Gag-TAP-associated protein samples under the strict condition in which the TAP-only lane had no observable band. In accordance with the reviewer's request, we have added detailed information on the proteins identified from each band in **New Supplementary Table 1**. We also more precisely describe the procedure for IGD and peptide mapping analysis in the **New Figure 1a** and the Materials and Methods section (**Page 19, Line 2**).

3-1. Figure 2: Did the authors use full-length APC fusions? If not, please explain.

<u>Response:</u> We did not use the full-length APC protein since it was quite difficult to synthesize in a wheat cell-free system due to its larger size (> 300 kD). We explain this issue in our revised text (**Page 6, Line 4**).

3-2. Also, based on the cartoon in panel A, it looks as though the GST tag is fused to the N-terminus of MA, which might be expected to perturb its folding. Please address this issue.

Response: We appreciate the reviewer's insight. Indeed, we used Gag proteins fused with GST at the N-terminus of MA in our binding experiments. As per the reviewer's suggestion, we newly created all Gag proteins with a C-terminal GST-tag and repeated the experiments with these constructs. We obtained very similar results to those using N-terminal tagged Gag proteins. These new data have been provided in our revised Figures (**New Figure 2 and New Figure 4b**).

4. Figure 3: In panels a-d please show the virion immunoblots and include the Gag CA (p24), p41 and Pr55 bands on the blots. This is necessary to validate release values. Also, with the SW480 blot (panel b), the cellular Gag bands for the APC- lane are much less bright than for the APC+ lanes, which could readily explain for the lack of Gag protein release as virions. Please use a better gel. I would also note that the top of the APC- blot in panel b looks highly contrasted. Perhaps a better blot here would be in order too.

Response:
1) We fully agree with this request and accordingly have included full size immunoblot figures that include the Pr55, p41 and p24 bands (**New Figure 3**).
2) We apologize for the poor quality of the SW480 blot. **New Figure 3b** now includes a higher quality image.

5-1) Figure 4 (= New Figure 5)*: Panel A: I do not see a significant difference between the examples of diffuse and PM staining. Better examples should be used. The authors also should use less subjective measurements of diffuse, PM and PM+Int staining.*

Response: We apologize for the poor quality of the figure in question. We have improved it in the revised paper (**New Figure 5a**). Quantitative analysis of subcellular localization of Gag-GFP is rather well established and has already been used in many other studies (e.g. Neil et al, PLos Pathogens, 2006; Miyakawa et al, PLos Pathogens, 2009). Briefly, 20 random fields of HeLa cells expressing HIV-1 Gag-GFP protein were inspected, and we counted the numbers of cells in which Gag-GFP was observed as punctate fluorescence at the plasma membrane (PM) only, as diffuse cytoplasmic fluorescence only, or at intracellular sites as well as at the PM. Enumeration of cells with each pattern of Gag-GFP localization was expressed as a percentage the total of the cells counted. The data we provide are representative of three separate experiments. We explain this in more detail in the Materials and Methods, citing the aforementioned references, in our revised text (**Page 22, Line 8**).

5-2) Panel B: I don't find the FRET data convincing.

Response: We acknowledge this concern. In general, for most cell biological applications, FRET exhibits a low signal to noise ratio due to the fluorescent properties of the YFP/CFP tags. To more precisely monitor intracellular Gag-Gag interactions, we conducted a recently developed NanoBRET (nano-bioluminescence resonance energy transfer)-based protein-protein interaction assay, which uses NanoLuc Luciferase as the BRET energy donor and HaloTag protein labeled with the NanoBRET 618 fluorophore, as the energy acceptor to measure the interaction of specific protein pairs (Machleidt et al., ACS Chem Biol., 2015). We found that the depletion of APC indeed reduced the NanoBRET signal based on the Gag-Gag interaction. These new data are now shown in **New Fig. 5b** and the procedures are described in the Materials and Methods in our revised text (**Page 23, Line 13**).

5-3) Panel C: Please show the entire virion blot.

Response: As suggested, we now include full size virion immunoblots (**New Figure 5c**).

5-4) Panel D: Did the authors score for p24 levels at later timepoints? If so, what were the results?

Response: We have added data for "15 days post infection" in the same experiment (**New Figure 5d**).

6. Figure 5 (= New Figure 6)*: Assuming that "APC targets cellular mRNA to actin-rich plasma membrane protrusions" (page 9), the vRNA localization is likely to be independent of the cis-active vRNA control elements. A control 24xMS2 RNA expressed from a non-retroviral plasmid ought to be tested. It also would be useful to graph cumulative RNA (MS2-GFP) line plots (summed from 20 or so images of each type) for quantification purposes.*

Response:

1) We fully accept this as a valid comment. In accordance with the reviewer's suggestion, we have generated a non-retroviral plasmid encoding firefly luciferase fused with 24xMS2-binding sites (pLuc-MSx24) (Jang et al., RNA Biol., 2016) and investigated its subcellular localization. We did not see any differences in its localization even in the presence or absence of APC expression, suggesting that APC does not affect the non-retroviral RNA with MS2-binding sites. These new data have been added as **New Supplementary Figure 3**.

2) Due to differences of the sizes and shapes of the cells, we could not overlay the line

plot data derived from different cells. Instead, we added another representative example of cell images (**Supplementary Figure 2c**). We also provide a bar chart indicating the percentage of cells with vRNA at the PM ($n > 20$) (**New Figure 6e**).

7. Figure 6-7: I think the data from these VS model are not so convincing, and that the figures and their discussion can be deleted.

<u>Response:</u> We agree that removing Figure 6 would not substantially diminish the impact of our study. However, we feel that Figure 7 might provide insights into the cell-to-cell spread of HIV-1, which we do consider to be an important point in our study. Therefore, we have improved Figure 7 in our revised manuscript. Please also see our response to Reviewer #2 on this issue below.

8. Supplementary Figure 3: An analyses of Fyn(10)WT, Fyn(10)6A2T and Fyn(10)RKswitch does contribute to the manuscript, but instead of using the alphascreen, and colocalization studies, the authors should simply perform parallel studies to Figure 3a and include the results as a figure in the paper itself.

<u>Response:</u> We fully accept this as a valid comment. In accordance with the reviewer's suggestion, the data in original Sup Fig 3 are now shown in **New Figure 4**. Moreover, we performed a HIV-1 production assay and AlphaScreen assay using WT, 6A2T, and RKswitch mutants (**New Figure 4a, 4b**). The results of these assays indicated that APC could interact with WT and RKswitch (both of which have highly basic residues in Gag), resulting in the enhancement of HIV-1 production. These results are in line with our conclusions in the original version of the manuscript.

9. Discussion: Assuming Figures 6-7 are deleted, the Discussion should be re-written to reflect this.

<u>Response:</u> We have modified the Discussion accordingly.

Responses to the comments of Reviewer #2

We sincerely appreciate the helpful and constructive suggestions by this reviewer. Reviewer#2 also regards this as an interesting study and has indicated that our current data define the role of APC as an important new host factor participating in the polarized localization of viral assembly to the site of cell-cell contact during virological synapse formation. This reviewer regards our experiments to have been well performed

and is supportive of our overall hypothesis. However, this referee does have concerns that epithelial cell lines were predominantly used in our study and has suggested that the manuscript would strengthened by experiments that show whether APC is expressed in primary T cells, and whether a knock down or knock out of APC in primary T cells has an effect on HIV-1 infection and cell-to-cell spread. We have responded to the concerns of Reviewer #2 below.

1) Would be important to evaluate the levels of endogenous APC in primary T cells and determine whether this interaction occurs within infected primary T cells.

Response: We accept this as a valid point. Accordingly, we confirmed the substantial expression of endogenous APC in primary CD4+ T cells (**New Figure 5e**). We also confirmed the pivotal function of endogenous APC in HIV-1 replication using primary CD4+ T cells via an shRNA-mediated knockdown experiment (**New Figure 5e**). To examine the endogenous APC-Gag interaction in HIV-infected T cells, we performed immunoprecipitation analysis using a latently HIV-1 infected CD4+ T cell line, ACH2, following treatment with PMA to activate virus expression. Consequently, we found that HIV-1 Gag was immunoprecipitated with endogenous APC in the CD4+ T cells (**New Figure 1e**). Moreover, we performed a cell-to-cell virus transfer experiment using HIV-infected primary CD4+ T cells as donor cells and LuSIV cells as the acceptor reporter cells for the detection and quantitation of HIV-1 replication (Roos et al., Virology, 2000) (**New Figure 7f**). Consequently, we found that targeted depletion of APC in the HIV-infected primary CD4+ T cells prominently reduced the cell-to-cell virus transfer. These new data together support our principal contention that APC promotes virus assembly and spread in CD4+ T cells.

2. Fig. 7: It is not clear whether the analysis is rigorously excluding cell-cell doublets. The study may also benefit from making stable cell lines that are expressing shRNA or have a deleted APC gene with CRISPR. It would be convincing to show that the levels of APC protein are lower in the presence of siAPC vs siCtrl.

Response: We acknowledge the reviewer's concern here. We excluded cell-cell doublets by detecting disproportions between cell size vs. cell signal in order to analyze the exact efficiency of viral transfer. Our new flow cytometry data are now presented in **New Figure 7d and 7e**.

In accordance with the reviewer's request, we performed a cell-to-cell viral transfer assay using primary human CD4+ T cells stably expressing APC-targeted shRNA via a lentivirus vector. We confirmed that the shRNA-mediated APC knockdown in T cells significantly decreases the cell-to-cell viral transfer. We also found that the level of APC protein is prominently lower in the presence of shAPC as compared with shCtrl cells.

These new data are included as **New Figure 7f**.

Responses to the comments of Reviewer #3

We deeply appreciate the careful analysis and constructive suggestions made by this reviewer. This reviewer was convinced by our experimental strategy and results but had concerns relating to the potential physiological relevance of Wnt signaling in APC-mediate HIV-1 assembly. As requested, we have revised the manuscript to address these concerns as indicated below.

1) It is formally possible that effects of APC gain or loss of function experiments on HIV assembly are due to APC´s role in Wnt signaling, i.e. that they are mediated by b-catenin dependent signaling rather than or in addition to its interaction with gag. In such a scenario activation of Wnt signaling by APC knockdown or in APC mutated colorectal cancer cells would prevent HIV assembly while inhibition of wnt signaling by full-size APC would stimulate it. It should be tested whether simultaneous knockdown of beta-catenin or expression of dominant-negative TCF blocks the consequences of APC knockdown in some of the key experiments shown in Figs. 4-7. Similarly, effects of b-catenin transfection or treatment of cells with Wnt3a should be determined.

Response: We acknowledge these valid suggestions. Accordingly, we performed several new experiments to investigate whether Wnt/β-catenin signaling affects the function of APC on HIV-1 assembly and production.
1) We found that the expression of beta-catenin in 293T cells had no observable effects on the APC-mediated enhancement of HIV-1 production, whereas it could significantly induce TCF4-dependent transcriptional activity (**Supplementary Figure 1a**).
2) We also found that the expression of dominant-negative TCF4 in SW480 cells (APC-mutated colorectal cancer cells) had no significant effects on HIV-1 production but reduces TCF4-dependent transcriptional activity (**Supplementary Figure 1b**).
From these results, we conclude that Wnt signaling does not directly contribute to the role of APC in HIV-1 production.

2 Please explain briefly what the pNL4-3 vector encodes for.

Response: We apologize for not explaining this abbreviation. pNL4-3 vector encodes full-length HIV-1 genome derived from HIV-$1_{NL\text{-}4\text{-}3}$ molecular clone obtained from the NIH AIDS Reagent Program. Upon transfection, this clone directed the production of infectious virus particles in a wide variety of cells. We have added this information to **Supplementary Table 1.**

3 What is the purpose of the Fyn part in fig. 3d?

Response: In the absence of Gag MA domain, which is essential for Gag membrane binding, HIV-1 cannot bud from cells. Meanwhile, the addition of N-terminal 10-amino-acid sequence of Fyn kinase [Fyn(10)], which serves as a heterologous membrane binding signal, is sufficient to partially rescue membrane binding of MA-deficient Gag and hence the budding of MA-deficient virus (Chukkapalli et al., J Virol, 2008). To address whether the MA domain is important for the functional interaction of Gag with APC in the absence of the membrane binding defect, we used MA-deficient Gag conjugated with the Fyn sequence. We have addressed these issues briefly in our revised text (**Page 7, Line 10**).

4. Fig. 4c and d would fit better with Figure 3.

Response: We have added APC-knockdown experiments using primary CD4+ T cells, and confirmed that the endogenous APC plays role in the function in HIV-1 spread (**New Figure 5e**).

5. Fig. 6 This a collection of neat experiments. However, how can authors state that GAG, vRNA and APC are enriched in protrusion if one doesn´t see the rest of the cell? Along that line, is it possible that absence of Gag and vRNA at protrusion after APC knockdown simply reflects lack of membrane association as shown for vRNA in fig. 5?

Response: We appreciate that this is a valid concern. As suggested by both reviewer #1 and the editor, we have excluded the original Figure 6 (trans-well assay data) from our revised manuscript. Since APC is predominantly localized at the cell protrusion or cell-cell contact sites rather than the rest of the cell body, we believe that APC is important for the accumulation of Gag and vRNA at such cell protrusion sites to enhance the efficiency of viral release/spread. These data will be further explored in our future studies.

Concerns of the Editor

Furthermore, there is an inconsistency in Figure 7a and 7b that needs to be addressed. The y-axis labeling suggests that "relative amount of Gag/vRNA at cell-to-cell contact site" is shown, while the figure legends suggests that "percentage of cells that Gag/vRNA was found at the cell-cell contact site" is shown.

Response: We apologize for this inconsistency and have amended our description in the

figure appropriately (**New Figure 7c**).

Other issue

1) We have newly added Masaki Anraku as a co-author of this paper since he conducted the FACS analyses to rigorously exclude cell-cell doublets in the cell-to-cell virus transfer assay. We confirmed that all co-authors have admitted it.
2) We found that the sequence of Fyn(10)6A2T shown in Old Supplementary Figure 3 contained an minor error, which originated from the error in previous paper by Llewellyn et al (J Virol 87, 6441, 2013). The correct 6A2T sequence is AWEAIALAPGGAAQYTLT (the positions of Thr are different), which is shown correctly in the supplementary figure in a former paper by Chukkapalli et al (PNAS 107, 1600, 2010). We have corrected this accordingly in New Figure 4a in our revised manuscript. This should not change any conclusion in the manuscript of our previous papers, but we are very sorry for the confusion that we made.
3) Words of the title were changed from “via interacting” to “via interaction” in order to make it clear.

REVIEWERS' COMMENTS:

Reviewer #1 (Remarks to the Author):

On the whole, the revisions incorporated into the Miyakawa et al. manuscript have improved the paper so that it should be of interest to Nature Communications readers. Although in some cases the effects of APC perturbation on virus production appears to be small, the authors in general have done a good job at quantification. I have only a few minor points of concern:
1. Supplementary Table 1: The authors show only single proteins identified for each of the cut out bands. I find it surprising that additional proteins were not identified in the MS sequencing. If other proteins were identified, even if their spectral count numbers were less than those of the proteins listed, they should be included in the table.
2. Supplementary Table 2: I still find the information on the constructs inadequate. In a supplement, it should not be difficult to show cartoon maps of the plasmids (which the authors presumably have), and indicate exactly what the junction sequences are.
3. Figures 5d-e: Full size Gag blots of the 15 day harvests similar to that shown in Figure 5c would be welcome.
4. Figures 5a and 6d: The analyses of these fluorescent images still borders on subjective, making the conclusions based on a very small number of images, or on subjective criteria as to the definitions of image classes.

Reviewer #2 (Remarks to the Author):

The revised manuscript by Miyakawa shows new data in primary T cells that knocking down APC reduced the efficiency of cell-cell transmission. While the magnitude of the influence of knockdown on viral transmission is modest, the data overall remains supportive of the role that APC is important in virus assembly and cell-cell transmission of HIV. Also improved in this manuscript is the presentation of the flow cytometry data that addressed issues of doublet discrimination. Overall the manuscript is improved, appears to be technically sound and with new data adequately addressed the concerns of this reviewer.

Reviewer #3 (Remarks to the Author):

The major concern of this reviewer was that APC´s effect on viral budding might be related to its role in wnt signaling rather than or in addition to its binding to gag. The authors show that neither activation of the pathway by b-catenin nor inhibition by dnTCF affects viral budding excluding such a scenario. All other issues were also addressed appropriately so that there are no further remaining criticisms.

As a minor correction the legend to Supplementary Fig. S1c,d should be changed from "Note that the expression of dnTCF4 in SW480 cells (APC-mutated colorectal cancer cells) had no observable effects on the APC-mediated enhancement of HIV-1 production, whereas

it significantly reduced TCF4-dependent transcriptional activity.”

to

“Note that the expression of dnTCF4 in SW480 cells (APC-mutated colorectal cancer cells) had no observable effects on HIV-1 production, whereas it significantly reduced TCF4-dependent transcriptional activity.” because the APC-mediated enhancement of HIV production was not studied in conjunction with dnTCF in this experiment.

Responses to the comments of Reviewer #1

On the whole, the revisions incorporated into the Miyakawa et al. manuscript have improved the paper so that it should be of interest to Nature Communications readers. Although in some cases the effects of APC perturbation on virus production appears to be small, the authors in general have done a good job at quantification. I have only a few minor points of concern:

Response: We sincerely appreciate the helpful and constructive suggestions by this reviewer. We have addressed the concerns raised by this reviewer as follows:

1. Supplementary Table 1: The authors show only single proteins identified for each of the cut out bands. I find it surprising that additional proteins were not identified in the MS sequencing. If other proteins were identified, even if their spectral count numbers were less than those of the proteins listed, they should be included in the table.

Response: According to the reviewer's request, we have included several other proteins with lower spectral count numbers in **New Supplementary Table 1**.

2. Supplementary Table 2: I still find the information on the constructs inadequate. In a supplement, it should not be difficult to show cartoon maps of the plasmids (which the authors presumably have), and indicate exactly what the junction sequences are.

Response: This is a valid comment for readers. We have added the cartoon map with junctional linker sequences of newly created plasmids (**New Supplementary Table 2**).

3. Figures 5d-e: Full size Gag blots of the 15 day harvests similar to that shown in Figure 5c would be welcome.

Response: As suggested, we have added the full size Gag blots in **New Figure 5d-e**.

4. Figures 5a and 6d: The analyses of these fluorescent images still borders on subjective, making the conclusions based on a very small number of images, or on subjective criteria as to the definitions of image classes.

Response: We analyzed the subcellular localization of Gag-GFP at least 100 randomly selected cells. Also, we analyzed vRNA localization at least 20 cells in randomly selected fields in confocal images. We added the detailed descriptions in our revised text (**Page 9 Line 3; Page 11, Line 1**)

Responses to the comments of Reviewer #2

The revised manuscript by Miyakawa shows new data in primary T cells that knocking down APC reduced the efficiency of cell-cell transmission. While the magnitude of the influence of knockdown on viral transmission is modest, the data overall remains supportive of the role that APC is important in virus assembly and cell-cell transmission of HIV. Also improved in this manuscript is the presentation of the flow cytometry data that addressed issues of doublet discrimination. Overall the manuscript is improved, appears to be technically sound and with new data adequately addressed the concerns of this reviewer.

Response: We wish to express our highest appreciation to Reviewer#2 for his/her insightful comments, which have helped us significantly improve our manuscript.

Responses to the comments of Reviewer #3

The major concern of this reviewer was that APC's effect on viral budding might be related to its role in wnt signaling rather than or in addition to its binding to gag. The authors show that neither activation of the pathway by b-catenin nor inhibition by dnTCF affects viral budding excluding such a scenario. All other issues were also addressed appropriately so that there are no further remaining criticisms.

As a minor correction the legend to Supplementary Fig. S1c,d should be changed from "Note that the expression of dnTCF4 in SW480 cells (APC-mutated colorectal cancer cells) had no observable effects on the APC-mediated enhancement of HIV-1 production, whereas it significantly reduced TCF4-dependent transcriptional activity." to "Note that the expression of dnTCF4 in SW480 cells (APC-mutated colorectal cancer cells) had no observable effects on HIV-1 production, whereas it significantly reduced TCF4-dependent transcriptional activity." because the APC-mediated enhancement of HIV production was not studied in conjunction with dnTCF in this experiment.

Response: We appreciate the valuable comments from Reviewer#3 and believe that our manuscript has been improved by his/her constructive suggestions. We acknowledge these valid suggestions regarding the figure legend of Fig. S1c,d. We have modified the legend according to the reviewer's indication.